Effect of physiological caffeine concentration on isolated skeletal muscle force, power and fatigue resistance

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Effects of Physiological Caffeine Concentration on Isolated Skeletal Muscle Force, Power and Fatigue Resistance

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Dedication

I would like to dedicate this work to my beautiful fiancé and soul mate Hayley Macdonald, without her love and support none of this would have been possible. Thank you for believing in me and agreeing to stand by me till the end of time.

Also to my grandmother Janet Everton, for being a constant source of motivation and encouragement throughout my life. Your pride inspires me to work to the best of my capabilities.

To my parents Simon Tallis and Julie Mcgann, thank you for giving me the freedom and opportunity to pursue my own interests and teaching me that there is no substitute for honest hard work. I am an extension of both of you and proud of it.

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Abstract

Caffeine is the most widely consumed socially acceptable drug in the world and is commonly used for its ergogenic properties with demonstrated performance enhancing effects in endurance, power and strength based activities. Despite a wealth of evidence concluding a caffeine induced performance benefit, the direct effects of the drug on peripheral physiological processes have not been fully examined. Early works showed high dose caffeine has direct force potentiating effect on skeletal muscle, a notion that has only recently been confirmed by James *et al.* (2005) to also occur at a maximal human physiological concentration (70μM). The present research, using mouse muscle as a model for mammalian muscle in general, provides an in-depth assessment of the direct effect of physiological concentrations of caffeine on isolated skeletal muscle performance. This research uniquely: quantifies the dose response relationship; assesses the effects of caffeine on maximal and sub maximal muscle power output and fatigue; looks at the relationship between muscles with different fiber type compositions. As high concentrations of caffeine and taurine are a constituent of many energy drinks, the suggested interaction of these ingredients to further potentiate muscle mechanical performance was also assessed. The study further examines how mammalian muscle mechanical properties change over an age range of development to aged, and how this differs between muscles with predominantly different anatomical locations and functions. In light of this the age related direct effect of physiological concentrations of caffeine was assessed in order to examine whether the ergogenic benefit changed with age. The present results demonstrate a direct muscle performance enhancing effect of physiological concentrations of caffeine that is likely to promote greater benefit on long duration endurance based activities. Furthermore, the present study demonstrates that there is no direct effect of physiological concentrations of taurine and no further performance enhancing benefit when combined with caffeine. Finally this research uniquely highlights the muscle specific age related changes in mechanical performance and further indicates that the direct effect of caffeine changes with age.

Presentation of the Results

Results of the present thesis have been published in peer reviewed journals, presented at international conferences and featured in the world media as follows (at the date of print):

Chapter 4 - The Effect of Physiological Concentrations of Caffeine on the Power Output of Maximally and Sub Maximally Stimulated Mouse EDL (Fast) and Soleus (Slow) Muscle

- Published in The Journal of Applied Physiology (see reference Tallis *et al.* 2012)
- Presented, in part, at The Society of Experimental Biology annual meeting in July 2010 and July 2011
- Published article in the World's media in July 2010

Chapter 5 - Does a Physiological Concentration of Caffeine (70μM) Affect Endurance in Maximally or Sub Maximally Stimulated Mouse Soleus (Slow) muscle?

- Published in The Journal of General Physiology Tallis, J., James, R.S., Cox, V.M., and Duncan, M.J. (2013) 'The effect of a physiological concentration of caffeine on the endurance of maximally and submaximally stimulated mouse soleus muscle.' *The Journal of Physiological Sciences*

Chapter 8 - Does the Ergogenic Benefit of Caffeine Change with Age? The Effect of Physiological Concentrations of Caffeine on the Power Output of Maximally Stimulated Mouse EDL and Diaphragm Muscle

- Presented at The Society of Experimental Biology annual meeting in July 2012
- Published article in the World's media in July 2012

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Abbreviations

A_{2a}	Adenosine Receptor 2a
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
Ca^{2+}	Calcium ion
CaP_i	Calcium Phosphate
Cl^-	Chloride ion
CK	Creatine Kinase
CNS	Central Nervous System
CSA	Cross-Sectional Area
EDL	Extensor Digitorum Longus
FFA	Free Fatty Acid
GH	Growth Hormone
H^+	Hydrogen ion
HFF	High Frequency Fatigue
IGF-1	Insulin like Growth Factor 1
K^+	Potassium ion
$kN.m^{-2}$	Kilonewtons per square meter
LFF	Low Frequency Fatigue
mM	Millimolar
µM	Micromolar
Na^+	Sodium ion
PO	Power Output
Pi	Phosphate ion
RER	Respiratory Exchange Ratio
ROS	Reactive Oxygen Species
RYR	Ryanodine Receptor
SE	Standard Error of the mean
SD	Standard Deviation
SR	Sarcoplasmic Reticulum
V_0	Maximum unloaded shortening Velocity
$V0_2$	Volume of Oxygen consumption
WADA	World Anti-Doping Agency
$W.kg^{-1}$	Watts per kilogram
WL	Work Loop

1. Introduction

1.1. General Introduction & Thesis Outline

Caffeine is the most commonly consumed socially acceptable drug in the world, a constitute of common drinks such tea, coffee, sodas and energy drinks, it is consumed largely for its stimulant and ergogenic effects (Graham, 2001). It is estimated that approximately 80% of the world's population consumes a caffeinated product every day, and that 90% of adults in North America consume caffeine on a daily basis (Ogawa & Ueki, 2007; Heckman *et al.* 2010).The performance enhancing properties of caffeine have been evidenced over a broad range of endurance and high-intensity based exercise activities (Graham, 2001; Davis & Green, 2009, Astorino & Roberson, 2010). On the basis of this evidence, caffeine has become a lucrative marketable ingredient and is the key component of many energy drinks (Reissig *et al.* 2009). In the U.S. alone from 2002 to 2006, the average annual growth rate in energy drink sales was 55% with a 2006-estimated market value of $5.4 billion (Reissig *et al.* 2009).Despite a large body of research, the ergogenic effect of caffeine is still a widely investigated and current research area as scientists strive to improve our understanding of the effects and actions of this drug. The recent boom in the energy drinks market, and the removal of caffeine from the World Anti-Doping Agency's (WADA) prohibited list, has produced an era in which caffeine abuse is readily acceptable within sporting circles and therefore it is important to develop our understanding of its effects.Chester and Wojeck (2008) concluded that the use of caffeine amongst British athletes was more widespread and accepted in competitive sport particularly at the elite level following the WADA 2004 change in stance.

Once ingested caffeine is rapidly absorbed into the blood and its hydrophobic nature allows free passage across all biological membranes resulting in distribution to all tissues of the body (Magkos & Kavouras, 2005). The potential central and peripheral action of caffeine has been discussed in recent reviews, however the specific magnitude of response in each physiological system cannot be quantified separately via human studies due to the potential of caffeine to act on many major

physiological systems simultaneously and the ethical restrictions imposed on human testing (Graham, 2001; Magkos & Kavouras, 2005).The primary aim of the present thesis is to provide an in-depth evaluation of the direct effect of caffeine, at concentrations that are physiologically relevant to humans (70µM maximum), on isolated skeletal muscle performance. i.e. the aim is to determine whether physiological concentrations of caffeine can enhance muscle performance independently of the central effects of caffeine. Early work on isolated skeletal muscle suggested that high, millimolar, concentrations of caffeine (that would be toxic for human consumption: Fredholm *et al.* 1999) elicit a direct potentiation of contractile force by enhancing the process of excitation contraction coupling (Luttgau & Oetliker, 1967; Huddart, 1968; Weber & Herz,1968; Endo *et al.* 1970; Allen *et al.* 1989; Allen & Westerblad, 1995). More recently James *et al.* (2005) was the first to conclude a direct effect of 70µM caffeine (the maximum likely human physiological caffeine concentration) to promote force-potentiating effects in mammalian muscle, using isolated mouse extensor digitorum longus (EDL; relatively fast muscle). The work by James *et al.* (2005) provides the foundation for the present body of research where a more detailed evaluation of the effects of physiologically relevant concentrations of caffeine is gathered.

In the thesis the mechanical properties of isolated skeletal muscle are gathered using a number of methods, however like James *et al.* (2005), the present research implements the work loop technique where skeletal muscle is assessed during cyclical length changes that are a closer approximation of *in vivo* muscle function than previous techniques(Josephson, 1985; James *et al.* 1996). Based on the theme of evaluating the response of isolated muscle to physiologically relevant concentrations of caffeine, the thesis is composed of 5 research studies answering a number of unique research questions:

1. **The Effect of Physiological Concentrations of Caffeine on the Power Output of Maximally and Sub Maximally Stimulated Mouse EDL (Fast) and Soleus (Slow) Muscle**

The aim of the initial study is to examine the effects of 70μM caffeine (human physiological maximum) on isolated mouse muscle with predominantly different muscle fiber types (i.e. fast vs. slow). Furthermore the study also looks to assess if the magnitude of the caffeine effect is increased if the muscle is stimulated at a sub maximal intensity with the hypothesis that caffeine would stimulate a greater SR Ca^{2+} release at a lower stimulation intensity. Finally this study aims to quantify the dose dependant effect of a range of physiologically relevant doses of caffeine.

2. **Does a Physiological Concentration of Caffeine (70μM) Affect Endurance In Maximally or Sub Maximally Stimulated Mouse Soleus (Relatively Slow) Muscle?**

James *et al.* (2005) reported that fatigue resistance in maximally stimulated mouse EDL muscle (relatively fast twitch) was not significantly affected when treated with caffeine. The second study is the natural progression aiming to assess the effects of 70μM caffeine on the fatigue resistance of relatively slow muscle. Furthermore this study also aims to assess if the effect of caffeine is altered when the muscle is stimulated at a sub maximal intensity. The aetiology of fatigue will be different between muscles with different predominant phenotype and when activated at maximal vs. sub maximal intensity. Therefore the potential link between aetiology of skeletal muscle fatigue and the ergogenic effect of caffeine can be established.

3. **Does a Physiological Concentration of Taurine Increase Acute Muscle Power Output, Time to Fatigue, and Recovery in Mouse Soleus (Slow) Muscle With or Without the Presence of Caffeine?**

In conjunction with caffeine, taurine is a further constituent of energy drinks that is included in large concentrations. Despite this the acute effect of taurine on performance has received very little attention. The present study aims to assess the potential direct effects of physiologically relevant concentrations of taurine on isolated skeletal muscle performance and to further investigate the

suggested hypothesis that taurine may act to increase the ergogenic effects of caffeine (Geib *et al.* 1994; Steele *et al.* 1990).

4. **Do Skeletal Muscles with diverse Functions Age at Different Rates? The Effect of Ageing on Isolated Locomotory (EDL) and Respiratory (diaphragm) Skeletal Muscle Performance**

The vast quantity of research publications that assess the ergogenic properties of caffeine on performance are conducted on subjects that are within an age range corresponding to their physiological prime. The effect of caffeine to promote performance-enhancing effects in children and the elderly is a sparse research area, and subsequently, a key area of interest that this thesis looks to address.

This study aims to: firstly provide detail of changes in skeletal muscle mechanics over a mammalian life span; secondly investigate if the ageing and development of muscles vary between muscles with differing function and composition (i.e. EDL muscle, a relatively fast twitch locomotory muscle under voluntary neural activation Versus diaphragm, a core muscle of mixed fiber type used to power respiration and primarily under involuntary neural activation);Understanding the age related changes in skeletal muscle's performance could provide an interesting insight into the physiology of ageing, not previously assessed using the work loop technique. Determining the potential muscle specific effects of ageing that occur overtime could aid in improving future exercise regimes to enhance the quality of life in elderly citizens.

5. **Does the Ergogenic Benefit of Caffeine Change with Age? The Effect of Physiological Concentrations of Caffeine on the Power Output of Maximally Stimulated Mouse EDL and Diaphragm Muscle**

The final study in this thesis aims to address the earlier question as to the effects of caffeine on isolated muscle over an age range spanning from development to elderly. The increase in muscle function during development and the later decline in aged muscle are related to a number of

interacting mechanisms that may affect the direct ergogenic properties of caffeine at the skeletal muscle level with primary consideration to changes in excitation contraction coupling and predominant muscle fiber type (Luff & Atwood, 1971; Navarro *et al.* 2001). With the direct effects of caffeine relating to excitation contraction coupling, a potential age related change in calcium handling would significantly modify the ergogenic benefit. As the development and ageing of skeletal muscle is likely to be related to compositional and functional properties, study five will assess the effects of caffeine on EDL and diaphragm muscle. Furthermore this study is the first to assess the effects of physiologically relevant concentrations of caffeine on diaphragm muscle.

1.2. *In vitro* Testing Methods for the Assessment of Isolated Skeletal Muscle Performance

In vitro testing is a procedure that takes place in a controlled environment on a non-living organism. The example used in the present research is the isolation of a muscle tendon unit via dissection and testing of the muscle properties, thus allowing exclusive examination of muscle performance (if the preparation is clamped at the tendons). In skeletal muscle testing, it is important that these control conditions correspond to the *in vivo* (real life) environment of the muscle(James *et al.* 2005).The *in vitro* method provides greater benefit in deducing biological mechanisms of action as isolation of a muscle in this way eradicates other possible contributors to performance. Such investigations into acute and chronic (fatigue) muscle performance, including studies by Askew *et al.* (1997), James *et al.* (2004), James *et al.* (2005), and Vassilakos *et al.* (2009), have implemented this technique in order to achieve these benefits allowing inferences to be made regarding skeletal muscle mechanisms. Krebs solution containing a supramaximal concentration of glucose manages limitations in muscle metabolism. Oxygen delivery as a variable is minimised, as the surrounding fluid is superfused with oxygen at a constant rate. The muscle is directly stimulated causing muscle contraction, therefore eradicating the effects of central stimulation; further to this the stimulation pattern given to the muscle is identical over time ensuring that any change in muscle force output is due to a change in the muscle mechanism of action. The environmental temperature can be maintained at a constant for all the preparations eradicating the effect of hypothermia and thermoregulation requirements. Rarely *in vivo* will a muscle produce a true maximal force due to central inhibition as a method to prevent damage (Macintosh, 2006). Therefore a maximal stimulation will produce a true maximal force output that may only be achievable *in vivo* in time of fight or flight. Furthermore it can be assured that during drug treatment trials, all muscle preparations are receiving the same dose throughout the duration of the experimental protocol as direct treatment eradicates difference in digestion that will occur in human consumption.

It is possible to test skeletal muscle properties *in vivo* using living organisms (Fulco *et al.* 1994), however there is greater difficulty in controlling confounding variables particularly in human based studies due to vast individual differences in genetics and lifestyle. The overriding downfall of *in vivo* testing is being unable to determine a clear division between central and peripheral factors contributing to performance.

Isometric, isotonic and isovelocity tests are common *in vitro* methods conducted to examine mammalian skeletal muscle properties. The work loop (WL) technique, originally explored in depth by Josephson (1995), has been more recently employed to provide a closer estimation of *in vivo* performance (James *et al.* 2004; 2005). A combination of such methods allows a profile of the contractile properties to be developed with these mechanical measurements providing information regarding the interaction of cross bridges (Fitts, 1994).

Simulating *in vivo* Performance for *in vitro* Testing

Temperature is a key variable that must be considered when testing muscle using any *in vitro* methods as this can have the greatest effect on the muscle's ability to perform work. This is widely established and discussed in a review by Marsh (1994), who concluded an increased activation and relaxation rate resulting in a power enhancement in different species of frog with increased temperature. For greater validity of results a physiologically relevant temperature must be used which is not the case in many early studies where low temperatures were used in order to keep the muscle alive for longer (Luttgau & Oetliker 1968).

To provide greater replication of muscle function *in vivo,* simulation of *in vivo* activation and length change cycles must be considered. These *in vivo* variables can be determined via electromyography to measure muscle activation, high speed video analysis or cine and sonomicrometery to determine muscle length changes (Coughlin *et al.* 1995).

Isometric Testing

The generation of muscle force relates to the number of actinomyosin cross bridges in force generating states (Rome, 2002). Isometric testing holds the muscle at a constant length throughout electrical stimulation allowing examination of the muscle's ability to produce maximum force whilst static. Commonly isometric twitch and tetanus experiments are conducted on the muscle. A twitch response represents the smallest contractile response that can be elicited (Figure 1.2.1.). The delay between the stimulation and response (Figure 1.2.1.) of the muscle is known as the electromechanical delay (latency period) and denotes the time required for depolarisation of the t-tubles, release of calcium into the intracellular space and the initiation of the cross bridge cycle (Cavanagh & Komi, 1979). Twitch magnitude and duration are greatly affected by the speed of Ca^{2+} release, Ca^{2+} affinity with tropnonin, and the speed of cross bridge cycling (Jones, 2004). The speed of these biochemical reactions is determined greatly by muscle fiber type and temperature (Macintosh, 2006).

A tetanus response occurs when the muscle is subjected to a number of subsequent electrical stimuli (Figure 1.2.1.), each occurring before the muscle is relaxed (Macintosh, 2006). In this case the muscle fibers are stimulated before relaxation of the twitch can occur, therefore the next contraction will produce greater force as the level of intracellular Ca^{2+} is elevated. This combination of twitches is known as summation (Macintosh, 2006). Elevating stimulation frequency will promote sustained force generation. An unfused tetanus displays partial relaxation between stimuli whereas contraction is sustained in a fused tetanus. The peak force achieved depends on the frequency and number of stimuli and consequently the concentration of Ca^{2+} release and the number of myosin cross bridges that can form. Prior to the final stimulus the muscle force remains high (Figure 1.2.1.), this is due to the high level of intracellular Ca^{2+}, and subsequent relaxation is dependent on the speed that Ca^{2+} can be removed from the sarcoplasm (Jones, 2004).

Figure 1.2.1 -Typical twitch and tetanus responses. Isometric tension in response to a single and a series of muscle action potentials. The dashed lines show the expected response if only the first action potential of a series occurred (Matthews, 2003)

In both twitch and tetanus methods maximal performance will occur at an optimal muscle length, so a number of tests should be conducted changing the length of the muscle and examining the responses. From this a force length curve (Figure 1.2.1.) can be developed highlighting this most favourable length where optimal thick and thin filament overlap occurs resulting in maximal cross bridging (Rome, 2002). The force length relationship was first established using length clamp experiments conducted by Gordon *et al.* (1966).

Figure 1.2.2.The force length relationship and single sarcomere myofilament interaction during shortening and lengthening (Matthews, 2003)

Largely isometric studies are useful for controlled investigation of a muscles maximal contractile force. James and Navas (2008) investigated the effect of temperature (15, 25 and 35^{o}C) on muscle performance in toads. The results indicated no significant change in maximal tetanic force production with increased temperature however a significant increase in the rates of force generation and relaxation.

A limitation to isometric testing is its lack of validity to real muscles that produce power during human movement *in vivo*. It is rare for muscle to be acting completely isometrically and usually shortening is required for the muscle to perform work (Rome, 2002). During *in vivo* locomotion dynamic activation is required and thus the muscle will often go through a large range of motion

resulting in altered muscle length. James *et al.* (1996) concluded that isometric testing vastly underestimated the *in vivo* rate of force activation and relaxation and is limited by not considering the passive properties of muscle. A muscle cannot shorten indefinitely and it will eventually have to re-lengthen. Therefore there will be a time *in vivo* where the muscle will be producing negative work (Josephson, 1993).

Isotonic Testing

Isotonic testing involves maintaining the muscle at a constant force and usually involves usage of Hill's (1938) equation to determine the force-velocity relationship of a muscle:

$$(F+a)(V+b)=(F_0+a)b$$

Where: F *is the tension (or load) in the muscle,* V *is velocity of contraction,* F_0 *is the maximum isometric tension (or load) generated in the muscle,* a *is coefficient of shortening heat,* $b = a \cdot V_0/F_0$*,* V_0 *is the maximum velocity, when* $F = 0$

Shortening velocity is one of the most predominant features in distinguishing between muscle fiber types. Important dynamic properties of active muscle can be evaluated by exploring the relationship between the muscle force output and velocity of shortening (Fig 1.2.3). The most important aspects of the force velocity relationship are the curvature of the line and the muscle's maximum shortening velocity (V_{max}; Barclay & Litchtwark, 2007). The hyperbolic force velocity curve denotes that the velocity of muscle contraction is inversely proportional to the load. During isotonic testing the muscle will shorten against a constant load and the shortening velocity of the muscle is plotted against resistive force. The theory behind the force velocity relationship relates to the sliding filament hypothesis and lack of time for cross bridges to form as shortening velocity increases. Further to this increasing shortening velocity will result in a decreased calcium sensitivity of the muscle (Hofmann & Moss, 1992). The force velocity relationship highlights a point of maximal power

output (PO) as a product of muscle force multiplied by velocity. This optimal level will rarely arise *in vivo* due to the varying shortening velocities and force production of the cyclical movement occurring (James *et al.* 1996). Josephson (1993) stated the importance of force velocity curves in the measurement of instantaneous power output but that force-velocity studies lacked the capability to provide information regarding sustainable PO.

When considering dynamic muscle power force production and shortening velocity will change, this is not considered in this method. James *et al.* (1996) concluded, from their study examining isometric and isotonic muscle properties as determinants of WL output in rabbit latissimus dorsi, that isotonic testing vastly overestimated muscle PO. The force velocity curve implies that a muscle can shorten indefinitely, as negative work is not considered PO calculated from this technique is approximately double that derived from the WL technique. In order for the power output predicted from force velocity data to be maintained through repetitive cyclical length changes, James *et al.* (1996) suggested that several unrealistic conditions would have to apply. The muscle must activate and relax instantaneously and muscle force and shortening velocity must remain at an optimal throughout shortening. As previously highlighted this is not an attainable feat during *in vivo* action as variation in muscle length and activation will alter the force produced. In conjunction with this shortening velocity will also change particularly when the muscle is coming to the end of shortening. The muscle should be fully relaxed throughout lengthening as lengthening against a resistance will increase the amount of negative work, therefore there should be no visco-elastic loss of energy.

Figure 1.2.3.– The isotonic force-velocity relationship of a typical mouse soleus muscle (Askew & Marsh, 1998)

Isovelocity Testing

Isovelocity testing involves shortening the muscle at a constant speed and has been used as a second method of attaining force-velocity relationships and a predictor of PO. Commonly this form of testing is achieved using the step-and-release protocol whereby isovelocity releases are immediately preceded by a step length change (Barclay, 1996). The muscle is initially stimulated to produce an isometric tetanus, however once peak force has been achieved a muscle length change occurs causing the muscle force to decrease. The muscle is then shortened from this new length at an estimated velocity that enables the muscle to maintain its new level of force. Animals have different fiber types, thus a different V/V_{max} and throughout movement will operate over a range of V/V_{max} (Rome, 2002).Measurement of shortening velocity again represents a single muscle

characteristic and although valuable it is not a true simulation of dynamic muscle contracture *in vivo* as muscle-shortening velocity will alter throughout a movement.

The 'iso' tests have the benefit of determining the intrinsic relationships between three basic muscle characteristics, force, length and velocity. Graphical representation from the data gained provides visual representation of the occurrences in active muscle. By using 'iso' methods much of the original theory behind how muscles operate during movement has been gained, as well as the underlying mechanisms. Although some muscles operate under these conditions, particularly isometrically contracting stabilising muscles, dynamic contraction integrates all of these factors and thus in this instance the limitations should be considered. Initially 'iso' testing ignores the passive properties of the muscle (James *et al.* 1996), a muscle is unable to shorten indefinitely and at some stage must return to its original length. Therefore the muscle must undertake negative work and requires energy to re-extend. Furthermore, muscle force, length, and velocity are dynamic factors throughout human movement and the interaction of these properties should be considered (Josephson, 1985). In addition these methods of testing do not accurately consider the time needed for muscle activation and relaxation, which further extends the limitations when relating the results to *in vivo* performance.

The Work Loop Technique

The current research primarily implements the work loop technique due to its greater validity in relation to muscle performance for *in vivo.* Previously outlined *in vitro* testing methods are poor estimates of true muscle function *in vivo* testing (James *et al.* 1996). Muscles work in antagonist pairs and go through cyclical length changes to produce a frequent movement necessary for locomotion (e.g. walking and running; Josephson, 1985). This practice is able to establish how much work and power is available from a muscle undergoing a cyclical length change.

The work loop technique was initially developed by Machin and Pringle (1959) as a method of investigating the mechanics of asynchronous flight muscle. Josephson (1985) proposed the advantages of the WL method over other *in vitro* techniques as being the first to consider the multiple factors that determine muscle performance; for example activation events, shortening dependant deactivation and pre-stretch effects.

Commonly sinusoidal length changes are used as estimates of muscle function during repetitive locomotory movements. Although it has been established that *in vivo* muscles will go through much more complex length change cycles this movement pattern has been considered a good, easy to implement, generalisation. The WL technique simulates the activity of the muscle by emulating this length change pattern and stimulating the muscle to contract. The muscle is subjected to the electrical stimuli at a selected time within the length change cycle; stimulation can be in the form of a single stimulus or a burst of stimuli. The area of the work loop represents the net work output per cycle; this is formed by plotting muscle stress (force ÷ cross-sectional area) against strain (length change ÷ initial length) over a full cycle (Van Leeuwen, 1992). The network generated is the difference between the energy required to stretch the muscle and the work produced during subsequent shortening (Josephson, 1985; Figure 1.2.4).

The major advantage of the WL technique is that it is the only *in vitro* technique that considers both the active and passive properties of the muscle (Josephson, 1985). For example a flexor muscle developing force during joint flexion and then relaxing to offer a small resistance to elongation during subsequent extension would produce a positive net energy output per cycle as the muscle has performed positive work on the moving joint (Figure 1.2.4; A). If however the muscle were inactive during flexion of the joint and active during extension to resist elongation net energy output would be negative, as the limb is required to perform work on the muscle. A muscle could be inactive throughout the whole of the length change cycle, producing passive net negative work (Figure 1.2.4; B). However positive work loops still have a passive element as the muscle cannot

shorten indefinitely and must be re-extended during each cycle involving a small amount of negative work. To minimise negative work the muscle must be relaxed during this elongation (James *et al.* 1996). However not all muscles may produce net positive work *in vivo* e.g. those acting eccentrically for braking and joint stabilisation are active whilst lengthening and consequently produce a net negative WL.

*Figure 1.2.4 – **A**: Construction of a net positive active work loop, **B**: Construction of a passive work loop (adapted from Josephson, 1985)*

Passive negative work is largely due to the connective tissue arrangement within the muscle, thus energy is required to stretch the muscle to overcome passive resistance to lengthening. In larger muscles the amount of connective tissue will be greater thus greater energy requirement will be need for elongation. The direction of the loop distinguishes between net positive and net negative work loops. Anticlockwise loops represent net positive work output by the muscle whilst net negative clockwise loops depict energy absorption or negative work. Average PO (J s^{-1}) can be

calculated by multiplying net work output per cycle (Joules) by cycle frequency (s^{-1}) (Josephson, 1993).

The WL technique has become a common *in vitro* method for examining the effects of muscular fatigue. Changes in the WL shape provide visual indication of a change in the muscle properties and its ability to produce positive work. Askew *et al.* (1997) used mouse soleus to investigate the effects of attainable cycle frequency on the development of fatigue using repeated WL cycles. During fatigue a reduction in force, a slowing in deactivation and changes in the force velocity properties of the muscle were reported. These mechanical changes were indicated by alterations in the shape of the work loops and also by highlighting an increase in negative work and a decrease in positive (Figure 1.2.5). Further to this greater cycle frequencies demonstrated a decreased ability to maintain muscle PO, however a midrange cycle frequency produced the highest absolute PO.

The initial and most obvious point to note in relation to WL shape during fatigue development is a reduction in the area of the WL thus indicating a reduction in muscle work (Figure 1.2.5). This reduction in work consists of a decrease in both the peak force attained and the force maintained during shortening. As fatigue develops it takes longer for this peak force to occur indicating a decreased activation rate. In conjunction with this, and evident to a greater degree, is prolonged relaxation apparent from the increased duration in the time required for the muscle to reach its baseline force (Vassilakos *et al.* 2009). Therefore, as fatigue develops the muscle is still active at the end of shortening thus requiring greater energy to elongate the muscle indicated by an increased force during lengthening, resulting a decrease in net positive work. As fatigue progresses a figure of eight WL will develop with the right hand side portion of the loop consisting of the positive work and the left hand side portion negative as a greater proportion of the length change cycle is conducted during active lengthening (James *et al.* 2004). Further fatigue then results in a full negative work loop as the muscle will undergo the same length change cycle with greater force produced during lengthening than during shortening.

Figure 1.2.5 – Effects of fatigue on work loop shape in mouse soleus (Askew et al. 1997)

Parameters that will affect the PO from a muscle include, the cycle frequency implemented, the strain (length change) imposed on the muscle, the trajectory of this strain, and the electrical stimulation phase and pattern (Josephson, 1993). Askew and Marsh (1997) reported that increasing the proportion of the cycle spent shortening will result in increased muscle power output over a range of cycle frequencies. In rare cases such as cardiac and some insect wing muscle, muscles will operate via a twitch contraction per cycle (Josephson, 1985), thus a single stimulus would be appropriate for estimating *in vivo* net work output. However, most mammalian muscle will operate under conditions requiring a burst of stimulation per cycle. The optimal stimulus phase to maximise PO is where activation begins towards the end of the lengthening half cycle to allow time for activation prior to shortening and faster force increase. Increasing muscle strain will maximise the duration in which electrical stimuli can be applied but will result in a complementary increase in energy required to elongate the muscle back to its resting length. As a consequence increasing strain will result in a greater net work up to an optimal, after which a decline will occur (Josephson, 1993).

In vitro muscle testing is subject to a vast number of parameters that can greatly affect mechanical performance. The primary factors include the environmental conditions and the maintenance of

these conditions overtime. It is possible to conduct such experiments on fiber bundles or single fibers but the present study uses whole muscle in order to account for not only changes in contractile properties but also the interaction of all other properties of the skeletal muscle. Subsequently, using a whole muscle allows better simulation of *in vivo* performance, however larger preparations have increased risk of the development of an anoxic core (Barclay, 2005). In light of this the present research will use relatively small mouse muscle allowing greater diffusion of oxygen to the muscle core and thus limiting the previously described effect.

Muscle Mechanics and Function *in vivo*

The primary function of skeletal muscle is to provide the work and power that causes movement. Not all muscle in the body works in a predictable manner and the integrated muscle tendon unit should be considered. The type of movement and the intensity of the activity strongly dictates the muscle specific function, i.e. it is possible to perform an isometric contraction by pushing against a wall however muscle function will vary greatly over dynamic cyclical contractions such as those used for locomotion. Although many muscles produce work under cyclical contraction (hence the importance of the work loop technique), co-activation of muscles has largely been seen as a way to provide stability around a joint or to perform precise slow movements, and in some instances result in eccentric contraction (Marsh, 1999). Muscles that are active during lengthening absorb energy and reduce economy. In some instances, such as the findings of Full and Stokes (1998) and unpublished work by Buhanan and Marsh (cited Marsh, 1999), muscles have been shown to absorb more work than they produce. In light of this, how well does *in vitro* testing of muscle mechanical properties relate to *in vivo* muscle contraction?

Usage of the work loop technique to optimise sinusoidal length change waveforms to mimic cyclical contractions has provided much information regarding muscle function (Josephson, 1993). In some instances, such as fish swimming and insect flight, sinusoidal length changes provide an accurate representation of *in vivo* muscle length change cycles (Coughlin *et al.* 1996; Chan & Dickinson, 1996).

Despite sinusoidal length trajectories being used regularly in research employing the work loop method (Altringham & Young, 1991; Askew *et al.* 1997; James et al. 1996; 1996, 2004; 2005; Vassilakos et al. 2009), this should be deemed an approximation of *in vivo* cyclical muscle activities (Dickinson *et al.* 2000). Further *in vitro* research suggests that other cyclical shapes cause a significant enhancement in muscle performance. Greater muscle power output has been shown using a non-symetrical 'sawtooth'-shaped cycle whereby the shortening phase is longer than re-lengthening allowing a longer stimulation duration and a more complete activation of the muscle, a more rapid rise in force due to an increased stretch velocity and an increase in the optimal strain amplitude (Askew & Marsh, 1997; Marsh, 1999). Askew and Marsh (1997) reported 5-10% higher power in mouse soleus and EDL using 'sawtooth' waveforms compared to sinusoidal work loops. Despite a greater power, 'sawtooth' length trajectories would still be deemed a very basic generalisation as *in vivo* the pattern of fiber stimulation and length change waveforms are likely to be manipulated throughout movement (Wakeling & Rozitis, 2005).

During level running of turkeys ankle extensor muscle length change was small in and tendon energy storage and recovery provided more than 60% of the total work of the muscle tendon unit (Roberts *et al.* 1996). It is considered that when forces are high during ground contact some muscles work almost isometrically to provide tension and allow effective storage of elastic energy (Marsh, 1999). Biewener *et al.* (1998) reported similar findings to that of Roberts *et al.* (1996) in steady speed hopping in wallabies, thus further suggesting the view that the tendon elastic saving of energy is an important mechanism to enable animals to locomote at higher speeds without large increase in metabolic energy expenditure.

Mouse EDL and soleus muscle were not chosen for use in the present body of research because they are likely to function under sinusoidal or 'sawtooth' length change trajectories *in vivo*; rather they were selected because they are two commonly examined mammalian muscles that are either predominantly fast or slow twitch. As with previous research the present thesis will use sinusoidal

length trajectories as an easy to understand simplification of the length change waveforms used in real life locomotion (Dickinson *et al.* 2000). Length change trajectories are likely to be muscle specific and implementation of a sinusoidal length change waveform provides a convenient and comparative framework for examining different muscles (Askew & Marsh, 1997).

1.3. Skeletal Muscle Fatigue

Skeletal muscle fatigue is one of the greatest limiting factors to sporting performance and is defined as a reduction in force generating capacity that results from prolonged activity (Fitts, 1994). A failure in the process of excitation contraction coupling will contribute to this reversible phenomenon occurring during prolonged and intense activity. The overall effect of fatigue is a consequence of central and peripheral physiological actions that reduce the muscle's functional capacity.

The aetiology of muscle fatigue is complex and is still not fully understood; however it is universally recognised that a single factor will not be the sole contributor, but that fatigue will result from a multitude of interacting effects. Combinations of acidosis, CNS and metabolic factors will contribute to a reduction in the ability of the muscle to maintain power output and repetitive cyclical movements (Allen *et al.* 2008).

Central Fatigue

Fatigue of the Central Nervous System (CNS) is an area that has caused great debate with conflicting literature presented regarding this phenomenon. The initial occurrence in the process of muscle activation is an efferent impulse sent through the nervous system resulting in an action potential in the target muscle. A disruption in this efferent impulse may contribute to fatigue by affecting motor unit activation.

Evidence that condemns the CNS theory of fatigue stems from what is now deemed a classic study by Merton (1954). Merton (1954) attempted to determine whether a decrease in CNS drive or intensity was responsible for a reduction in the capacity of a muscle to produce force. In order to accomplish this Merton (1954) superimposed a large electrical stimulation onto a maximal voluntary contraction of a fatigued muscle with the theory that if CNS effects were responsible for reduced muscle force production, then an added external stimulation would increase the force. Results failed

to demonstrate any increase in muscle force, consequently it was concluded that CNS deficiencies were not responsible for muscle fatigue.

In contrast to this it has been established that prolonged exercise can disrupt the cerebral cortex (Enoka, 2008). It is believed that in addition to impairing output to the active muscles, disruption of the cerebral cortex will also impede one's ability to accurately perform cognitive tasks. One of the mechanisms believed to contribute to this disruption is a disturbance in the balance of neurotransmitters; however it is unclear how severe these effects are. More established areas to consider are the effects of elevated core body temperature and hypoglycaemia that can occur during prolonged exercise. Hyperthermia has been shown to decrease the ability of the cerebral cortex to produce the required efferent motor activity (Todd *et al.* 2005). Hypoglycaemia could also have an effect, as the cerebral cortex requires glucose as a fuel for energy regeneration (Enoka, 2008). A further idea is that efferent impulses are unable to cross the synapses of adjacent nerve cells due to a decrease in acetylcholine, thus the signal is not passed on as it fails to cross the synaptic cleft. It is believed that failed transmission of impulses may also occur at the neuromuscular junction such that an action potential in the muscle cannot be initialised (Gardiner, 2001).

A further theory relates to the exercise induced accumulation of serotonin, a neurotransmitter associated with effects on arousal, sleepiness, lethargy and mood (Davis *et al.* 2000). Glycogen depletion results in increasing amounts of amino acids released from the working muscles. An example of this is Tryptophan, which primarily travels in the blood bound to albumin; a small free concentration is able to cross the blood brain barrier promoting the synthesis of serotonin. Further to this as greater FFA's are released from adipose tissue, this competes for albumin resulting in a greater free concentration of tryptophan thus greater synthesis of serotonin (Davis *et al.* 2000).

EMG studies are a popular technique that has been employed to measure the electrical activity of skeletal muscle. Lepres *et al.* (2001) investigated the effects of prolonged cycling (>5 hours) on neuromuscular fatigue and reported significant reductions in EMG activity in the vastus lateralis

muscle. Lepres *et al.* (2001) concluded a decreased neural drive in the latter stages of exercise. Vandewalle *et al.* (1991) also demonstrated the occurrence of central fatigue after reporting decreased EMG activity in subjects using an adapted Wingate test. Kay *et al.* (2001) investigated neuromuscular changes during fatigue by implementing EMG during 6 high intensity 1-minute cycle sprints. Results gained by Kay *et al.* (2001) demonstrated that power output and IEMG decreased progressively during sprints 2-5, however both power and IEMG increased during the final sprint. In this instance fatigue was attributed to a combination of changes in neuromuscular recruitment and central or peripheral control systems.

Cellular Mechanisms of Fatigue – Skeletal muscle

Muscle fatigue can be subdivided into two categories, low (LFF) and high frequency fatigue (HFF). HFF is that induced by intense activity, characterised by a rapid reduction in force that swiftly recovers, attributed to readily reversible ionic imbalances affecting muscle excitation (Favero, 1999). LFF is characterised as force reduction due to activities at lower stimulation frequencies, here recovery is prolonged. LFF is attributed to a wider variety of mechanisms including mitochondrial and metabolic disruptions (Favero, 1999). The intensity of the activity greatly influences the aetiology of fatigue, however further considerations regarding variables such as fiber type distribution and training state of an individual must be noted.

In vitro methods have provided evidence for mechanical changes in the muscle and have allowed physiological conclusions to be drawn with regards to fatigue mechanisms. Askew *et al.* (1997) used mouse soleus muscle to investigate the effects of attainable cycle frequency on the development of fatigue using repeated work loop cycles. Fatigue caused a reduction in force, a slowing in deactivation and changes in the force velocity properties of the muscle, as indicated by a change in the shape of the work loops including an increase in negative work and a decrease in positive work (Figure 1.2.3). The force generated at the muscles' peak length and the maintenance of force through shortening rapidly decreased. Fatigued muscle also failed to completely relax by the end of

shortening which in turn increased the work required for muscle lengthening(Figure 1.2.3: Evident from work loop 20 at 2hz and 40 at 6Hz). Askew *et al.* (1997) also demonstrated that greater cycle frequencies caused a decreased ability to maintain muscle power output.

The importance of intracellular Ca^{2+} has already been explained; limiting the free Ca^{2+} concentration would result in decreased force production (Berchtold *et al.* 2000). At the onset a fatigue there is a reduction in myofibrillar Ca^{2+}sensitivity, meaning a reduction in the affinity for tropinin-C binding and consequently a reduced number of free actin binding sites for cross bridging (Allen *et al.* 1995). Fatigue also causes impaired Ca^{2+} release from the SR, thus reducing the effectiveness of the Ca^{2+} induced Ca^{2+} release mechanism (Allen *et al.* 1995). It has been established that the increased acidosis and accumulation of P_i associated with fatigue will reduce the sensitivity of myofibrils to Ca^{2+} (Godt & Nosek, 1988).

Acidosis

During prolonged high intensity exercise anaerobic glycolysis will become the primary method of ATP regeneration. A by-product of this form of metabolism is lactic acid and if production outweighs removal accumulation will occur (Powers & Howley, 2006). For many years it was believed that fatigue was associated with an accumulation of lactate, however it has now been established that the formation of H^+ ions associated with lactic acid production is not a large mediator in the onset of fatigue (Cairns, 2006).During anaerobic glycolysis H^+ is generated, such that in a high intensity bout of exercise the rate will be substantial and as an overall effect the tissue buffering system will be overcome. A net consequence of H^+ accumulation is a decrease in pH resulting in acidosis. Muscle pH at rest is maintained at 7.05; however, following intense activity this may drop as low as 6.5 (Allen *et al.* 2008). The fastest fiber type (IIb) will be more greatly utilized in high intensity activity thus this will be the site of greatest pH decline. The most widely established theory is that H^+ ions will compete with $Ca^{2+}$for binding sites on troponin-C, thus inhibiting binding of myosin heads and hence force production (Crains, 2006). Blanchard and Solaro (1984) reported a decrease in myofibrillar

troponin-C affinity for Ca^{2+} as pH was reduced, a conclusion further echoed by Palmer & Kentish (1994). A flux of H^+ has also been shown to inhibit the rate of cross bridge transition from weakly bound to a strongly bound state (Fitts, 2004). This reduction in strongly bound cross bridges will massively reduce the maximal force capacity of the muscle. High levels of H^+ are also believed to inhibit the SR calcium release channel and furthermore reduce the affinity of Ca^{2+} binding to the SR pump affecting relaxation (Allen *et al.* 2008). With Ca^{2+} not being removed as quickly from the intramuscular space, a larger concentration of free Ca^{2+} will occur resulting in greater isometric force production in early fatigue (Allen *et al.* 2008). High concentrations of free Ca^{2+} may inhibit the function of the t-tubules, with accumulation in this region blocking transmission of the action potential into the axial core of the fiber (Fitts, 1994).

Long-term high concentrations of Ca^{2+} within the muscle cytoplasm will cause an accumulation in surrounding organelles or movement of Ca^{2+} out of the cell. The result is a decline in free Ca^{2+} concentration in the muscle cytoplasm and ultimately that available for release from the SR. Lannergren *et al.* (2001) reported a marked increase in mitochondrial Ca^{2+} concentration in *Xenopus* fibers following repetitive tetanic contractions. However, the rate of Ca^{2+} extraction from the cell is believed to be very slow (Allen *et al.* 2008).

A change in muscle pH will decrease the activity of enzymes essential in energy regeneration and impairment of the myofilament proteins. The net result would be impairment of cross bridging activity, decreased Ca^{2+} activation of troponin and impaired function of myosin ATPase (Cairns, 2006). However a low pH could have beneficial effects on membrane conductance, it is believed that lowering the pH will reduce the Cl^- conductance of the t-tubular membrane allowing action potentials to propagate more readily (Allen *et al.* 2008).

A decreasing muscle pH is historically the most popular candidate for producing fatigue as it affects multiple sites. However, it has been established that pH can only explain a portion of the fatigue effect as the initial rapid stage that can be observed in recovery occurs when muscle pH is still

declining (Fitts, 1994). In recent years the relationship between lactic acid and fatigue has been reviewed by Cairns (2006). Correlation type studies provided the early evidence showing a relationship between H^+ accumulation and declining force. However in more recent studies using physiologically relevant experimental temperatures this negative effect is not shown at such an extent and in some cases acidosis has been shown to enhance performance (Fitts & Holloszy, 1976; Nielsen *et al.* 2001). The Cairns (2006) review reported that acidosis can have a protective effect against K^+ induced fatigue, however controversy exists about the mechanism.

However much the lactic acid hypothesis is scrutinised it is clear that acidosis plays a part in inducing fatigue during intense exercise bouts. Studies using H^+ buffering agents such as Sodium Bicarbonate have demonstrated that reduced acidosis will enhance exercise performance thus providing evidence for the acidosis theory. Lindh *et al.* (2008) reported that Sodium Bicarbonate ingestion improved mean200m freestyle swimming time by 1.5 seconds in elite male competitors. Sodium Bicarbonate studies *in vivo* produce mixed results. This subsequently highlights the need to examine the effects of sodium bicarbonate directly on skeletal muscle.

Inorganic Phosphate

A single molecule of ATP is hydrolysed during the cycling of each actin-myosin cross bridge. The splitting of the ATP molecule, via myosin ATPase, produces ADP and inorganic phosphate (Pi; Powers & Howley, 2006). Pi will also arise from ionic pumps in the muscle that work against a concentration gradient and thus require energy (i.e. Na^+-K^+ ATPase and Ca^{2+} ATPase). If ATP is metabolised at a greater rate than regeneration a resulting increase in free concentrations of ADP and Pi will occur in the muscle cytoplasm. In contrast to the buffering system in place to deal with high H^+ concentration, the regulation of Pi within the muscle is dependent on its transport into the mitochondria.

Pi at rest is maintained at 1-5 mM, however during intense activity this can increase to 30-40mM (Allen & Westerblad, 2001). In a number of studies a clear reduction in myofibrillar Ca^{2+} sensitivity due to elevated levels of Pi has been demonstrated (Allen *et al.* 1995). Elevated sarcoplasmic Pi concentration will promote fatigue by directly affecting the cross-bridge function, resulting in a decreased force during early fatigue. The release of Pi from the transition of cross bridges from a weakly bound to a strongly bound state suggests that Pi inhibits this process (Westerblad *et al.* 2002). Westerblad *et al.* (2002) further reported the inhibitory effect of Pi on the SR Ca^{2+}pump. Allen *et al.* (2008) stated that the mechanism results in a 10% decrease in maximal force at physiological temperature. It is also believed that accumulation of Pi will result in failure of Ca^{2+} release from the SR during fatigue and is the main contributor to an early fall in force during intense exercise.

The SR concentration of free Ca^{2+} is approximately 1mM, but the total concentration is much greater as it includes Ca^{2+} bound to binding proteins such as calsequestrin (Somlyo *et al.* 1981). Precipitation of calcium phosphate may occur as it is reported that Pi is capable of entering the SR via anion channels (Westerblad *et al.* 2002). Once the solubility product of Pi exceeds the 6mM threshold it binds to calcium producing CaPi and precipitation will slowly occur. CaPi precipitation causes a decrease in free SR Ca^{2+} concentration and that bound to calsequestrin therefore limiting its release into the intracellular space (Allen & Westerblad, 2001).

The value of Pi as a mechanism of fatigue can be viewed in a study by Dashlstedt *et al.* (2000) that genetically modified mice by inactivating creatine kinase, which prevents a rise in Pi due to inhibition of the catabolism of phosphocreatine. Here the early rise in force, increased sarcoplasmic concentration of Ca^{2+} and increased Ca^{2+} transient time are abolished leading to the conclusion that CK may contribute to muscular fatigue by resulting in an increased concentration of Pi.

Potassium

The membrane hypothesis of fatigue involves disturbances in excitation contraction coupling, altering sarcolemmal or t-tubular excitability (Fitts, 1994). Skeletal muscles' resting membrane potential is most reliant on potassium, such that any alteration in concentration gradient or conductance across the sarcolemma will vastly affect membrane potential.

A combination of the previously described ionic reactions to fatigue will result in an increased muscle osmolality. As well as impeding physical contraction of the muscle, increased osmolality will also account for up to a 50% increase in K^+ concentration depending on exercise intensity (Lindinger, 1995). During high intensity exercise a rapid force loss is accompanied by a slowing of action potential waveform, attributed to the accumulation of K^+ in the extracellular spaces of the muscle. This potassium theory generally dictates that a K^+ efflux, in conjunction with inhibition of the Na^+-K^+ pump or its capacity to regulate a K^+ efflux and a Na^+ influx, will result in cell depolarisation of the resting membrane potential (Lindinger, 1995). Changing the membrane potential will vastly limit the rate of firing action potentials and ultimately depressed t-tubular charge will result in inhibition of the SR Ca^{2+} release channel. The t-tubules in the interior of the muscle may depolarize enough to block action potentials, thus resulting in inactivation of the myofibrils (Fitts, 1994). These mechanisms can be viewed as a muscle safety instrument that protects the cell against Ca^{2+} accumulation and ATP depletion, preventing damage to the cell by avoiding catastrophic alterations in its homeostasis. The effect of fatigue on K^+ has also been established during prolonged activity with LFF being associated with a continued net loss of K^+ from the activated muscle (Lindinger, 1995).

Glycogen

During prolonged activity aerobic metabolism forms the basis of energy production; incidentally Pi and H^+ remain relatively low (Powers & Howley, 2006). The mechanisms of fatigue here are not

clearly understood however depletion of muscle glycogen and low blood glucose concentration are key determinants. Muscle recruitment for endurance-based activities largely involves type I and IIa muscle fibers, as these provide the greatest resistance to fatigue (Powers & Howley, 2006). A relationship between glycogen concentration and SR membranes in skeletal muscle has been established. It has been proposed that this association causes glycogen to regulate Ca^{2+} efflux from sequestration into the SR (Favero, 1999). Once the supply of glycogen is utilised beta-oxidation becomes the primary method of energy regeneration. Mobilising FFA's requires a greater quantity of oxygen to yield the same amount of ATP; here exercise intensity will decrease as the oxygen supply is limited.

Hypoglycaemia has also been shown to contribute to failure of the CNS; the store of glucose in neuronal tissue is limited and is therefore largely reliant on glucose from the bloodstream. Nybo (2003) investigated the effect of ingestion of carbohydrates during prolonged exercise and its effect on CNS activation of skeletal muscle. The study concluded that exercise-induced hypoglycaemia attenuates CNS activation during sustained muscle contraction.

Environmental Conditions

A well-established phenomenon is that prolonged exercise capacity will be greatly reduced in high environmental temperatures (Todd *et al.* 2005). The physiological stress caused by the environment will result in dehydration, thus a loss in plasma volume and a decreased venous return. Greater skin blood flow compromises muscle blood flow and therefore reduces substrate and oxygen delivery, as well as inhibition of by-product removal (Gonzalez *et al.* 1999).

A number of studies have demonstrated that a decreased ambient partial pressure of O_2, such as that at high altitudes, will significantly decrease time to fatigue (Fulco *et al.* 1994). A decrease in PO_2, and subsequent reduction in blood O_2 and its delivery to the working muscles, will severely reduce the rate of ATP regeneration; therefore exercise cannot be maintained.

Modality of exercise and the environment will have the greatest influence on the mechanism of fatigue. Biochemical changes affecting the release and affinity of Ca^{2+} resulting in a loss of muscle activation is a primary mechanism of fatigue. These alterations can affect the pathway of excitation contraction coupling from stimulation to filament interaction. This review can be deemed brief in light of what is a complex series of events. Other areas should be considered such as a link between a rising level of free radicals and control and/or modification of Ca^{2+} regulation has been established (Favero, 1999). However it is clear that a number of contributing factors will result in muscle fatigue depending on an individual's level of fitness, exercise intensity and environmental stressors. A primary way to enhance one's ability to offset fatigue is through a specific training regime or via the use of ergogenic aids, specifically caffeine.

1.4. Caffeine as an Ergogenic Aid

The effects of caffeine as a powerful ergogenic aid have been examined for its effects both on the central nervous system (CNS) and directly on skeletal muscle (Graham, 2001). *In vivo* and *in vitro* studies have found enhancements in endurance exercise performance, power and fatigue recovery, accredited primarily to the effects on the CNS (Kalmar & Cafarelli, 2004). Until 2004 caffeine was recognised as a banned substance by the World Anti-Doping Agency (WADA). Urinary levels exceeding 12 $\mu g.ml^{-1}$ post competition were deemed illegal and consequently led to athletes being disqualified (Spriet & Howlett, 2000). Presently caffeine is still not included on WADA's prohibited list (Word Anti-Doping Agency, 2012) despite its documented popularity as a performance enhancer as a aiding training and in competition. It is essential to critically examine some of the key research surrounding the ergogenic properties of caffeine in order to assess the quality of the experiments conducted and their relevance to human performance. There are a number of potential modes of action for caffeine, however this report will provide greater focus on the direct skeletal muscle response due to the context of this investigation.

Caffeine Digestion and Concentration in Blood

Caffeine (common name for 1,3,7-trimethylxanthine; Fig 1.4.1) is present in the diet of most individuals, but is not an essential nutrient, nor beneficial to promoting health (Magkos & Kavouras, 2005). Subsequently, prior to 2004 ingestion of caffeine as a means for improving performance was deemed as drug abuse.

Following digestion caffeine is readily absorbed into the blood stream and can be measured for up to 5 hours post ingestion. Peak plasma concentration will occur between 30-60 minutes following ingestion (Lorist & Tops, 2003). Caffeine is hydrophobic in nature allowing it to pass freely across all biological membranes, thus it can be distributed through all tissues of the body (Magkos & Kavouras, 2005). Caffeine is also able to diffuse from the cerebral circulation across the blood brain barrier

entering the spaces in the brain tissue in sufficient quantity to promote pharmacological effects (George, 2000).

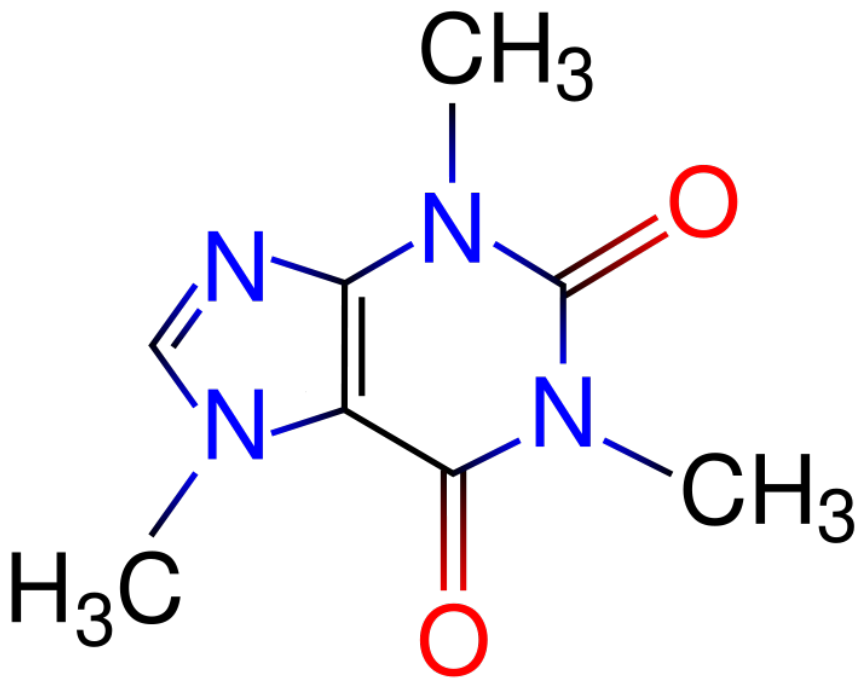


Figure 1.4.1. – The Chemical Structure of Caffeine

Caffeine is processed in the liver forming the three metabolites paraxanthine, theophylline, and theobromine (Goldstein *et al.* 2010). Caffeine and its metabolites are excreted through the kidneys and caffeine has a half-life of 2.5-10 hours in humans for a caffeine dosage below 10 $mg.kg^{-1}$. Elimination from the blood is dependent on the quantity consumed and the rate it is absorbed and metabolised (Magkos & Kavouras, 2005). As caffeine is able to cross all membranes it makes it increasingly difficult to determine where the greatest physiological effect occurs and it is misleading to assume that a single mechanism is responsible for the entire physiological response (Magkos & Kavouras, 2005).

Caffeine in Sport

Caffeine is a readily available, socially acceptable drug therefore high levels of consumption occur regularly within day-to-day life and the sporting world (Graham, 2001). Without a current limitation on the level of consumption within sport, a growing number of athletes are exploiting this method as a way of achieving a legal performance enhancement.

With the benefits of this ergogenic aid demonstrated within the scientific literature, the present marketing of caffeine as a performance enhancer has become a common occurrence with many sporting foods, drinks, tablets, gums and gels claiming to significantly improve performance (Davis & Green, 2009). Lucozade, the popular sports drink company, support the performance benefits of caffeine via their marketing of a caffeine boost drink that they claim has been proven to “enhance consistent work rate or running speed” (Lucozade, 2010). Also Red Bull energy drink claims to "increase performance, stimulate metabolism, and increase concentration and reaction speed (Red Bull, 2010). This represents a fraction of the high caffeine concentration products available on the market, all boasting similar claims.

Caffeine and Exercise Performance

Graham (2001) reviewed a multitude of studies investigating the influence of caffeine in a number of diverse exercise modes and intensities. In light of these findings it is possible to conclude performance enhancements occur in endurance (activity lasting greater than 30 minutes), power and strength activities, although conflicting verification does exist within these categories.

Table 1.4.1 represents a sample of the literature representing the effect of caffeine supplementation on endurance-based activities. Consistent with the reviews by Graham (2001), Davis & Green (2009) and Goldstein *et al.* (2010), the majority of evidence is in favour of an increased performance in the caffeine treated trials. In contrast Table 1.4.2 represents a sample of the literature regarding high intensity and muscular strength activities. Here the conflicting evidence suggests that a caffeine-

induced enhancement of performance is not as consistent and at least some of the diversity in these findings may be explained by the variety in testing methods. The majority of this evidence concerning human based studies results in an increased complexity in understanding the underlying locality of caffeine's greatest effect as in such whole body research a number of physiological systems are interacting to produce a change performance.

Bruce *et al.* (2000) represents a typical human based approach to the investigation of caffeine as a performance enhancer. This randomised, double blind, cross over study considered 2000m rowing performance in competitive athletes. 8 subjects conducted 3 trials after ingestion of a placebo, 6 or 9 $mg.kg^{-1}$ body mass of caffeine treatment 1 hour prior to participation in the exercise protocol. The low dose caffeine trial resulted in a significant 1.3% improvement in time to complete the 2000m, whilst the high dosage resulted in a significant 1% improvement. SD of ± 0.9% highlights a varied individual effect to the treatment, which is a common theme throughout such studies. An average mean power output increase of 2.7% was reported in comparison to the placebo trial. Again common in the vast amount of literature no significant differences occurred in HR, VO_2 or RER. Urinary samples following testing rose to 6.2 ± 3.6 $mg.l^{-1}$ for the low dosage and 14.5 ± 7.0 $mg.l^{-1}$ in the high dosage. The subjects received identical dosages of caffeine, yet there was a large SD in urinary samples following ingestion. With relevance to WADA's pre 2004 stand on caffeine competitors would be deemed cheating if urinary threshold exceed the 12 $mg.ml^{-1}$. However in some cases urinary sample may be lower for the same dosage of caffeine, as expressed in this study, thus revealing a problem in predicting how much caffeine will result in illegal urinary levels. Individuals will differ in caffeine uptake, rate of caffeine metabolism and excretion; this therefore will affect how much caffeine is required at an individual level to promote an enhancement in performance (Magkos & Kavouras, 2005).

Author	Subjects	Caffeine Dose	Protocol	Performance Enhanced	Comments
			Long Duration Endurance Activity		
Norager *et al.* (2005)	15 men and 15 women > 70 years – divided into group A (placebo trial then caffeine trial) and B (caffeine then placebo)	6 $mg.kg^{-1}$ 60 mins prior to exercise	Cycle ergometer with an increasing load of 25 W every second minute until 65% of the expected maximal heart rate was reached. The load remained at this level, and the time until exhaustion was recorded.	**YES**	Endurance significantly increased by 14% in *group A* and by 32%in *group B*
Graham *et al.* (1998)	9 actively endurance training males aged 27.8	4.5 $mg.kg^{-1}$ caffeine total volume of liquid 7.15 ml/kg 1 hour prior – caffeine in water, decaffeinated coffee with caffeine capsule or regular coffee	Ran at 85% of maximal oxygen consumption until voluntary exhaustion	**YES**	Endurance was only increased in the caffeine capsule trial (7.5-10 minutes). Increase in adrenalin in all 3 trials but no significant difference between trials. No treatment effects on blood glucose
Kovacs *et al.* (1998)	15 healthy and well-trained male subjects (triathletes and cyclists) 23.3 ± 0.9 yr	Drinking 14 ml/kg BW with 7% carbohydrate-electrolyte solution with 150, 225,and 320 mg/l caffeine	Cycle ergometer - perform a set amount of work (equal to 1 h cycling) as quickly as possible. (90 rpm at 70%)	**YES**	Completed the time trial significantly faster and with a significantly higher mean work output after ingestion of CES-225 and CES-320
Conway *et al.* (2003)	9 well trained cyclists and triathletes 25.5 ± 5	Compared effect of single and divided dose caffeine. Single caffeine dose 6 $mg.kg^{-1}$ 60 mins prior to exercise and placebo 45 into exercise divided caffeine dose 3 $mg.kg^{-1}$ 60 mins prior to exercise and 3 $mg.kg^{-1}$ 45 mins into exercise	Cycled for 90 min at 68% maximal oxygen uptake followed by a self-paced time trial (work equivalent to 80% of max oxygen uptake workload over 30 mins)	**Probable**	Tendency shown for faster time trial performance in the caffeine trials (P=0.08) caffeine and placebo 24.2 mins and divided caffeine trial 23.4 mins compared to 28.3 mins in the placebo trial. Study concluded divided dosage results in no added ergogenic effect
Bell & Mclellan (2002)	15 males and 6 females subjects 32 ± 7 yrs (mean±SD) 13 caffeine users and 8 caffeine non users	5 $mg.kg^{-1}$ caffeine 1,3 or 6 hours prior to testing	Cycling to exhaustion at 80% VO_{2max}	**YES**	Non-users, exercise times 1, 3 and 6 hours after caffeine 32.7 ± 8.4, 32.1 ± 8.6 and 31.7 ± 12.0 min, each significantly greater than the corresponding placebo values 24.2 ± 6.4, 25.8 ± 9.0 and 23.2 ± 7.1 min. Caffeine users, exercise times 1, 3 and 6 were 27.4 ± 7.2, 28.1 ± 7.8 and 24.5 ± 7.6 min, respectively. Exercise times 1 and 3 hours were significantly greater than the respective placebo trials of 23.3 ± 6.5, 23.2 ± 7.1 and 23.5 ±5.7 min.
Cohen *et al.* (2006)	5 men 2 women (23-51 years old)	0.5 or 9 $mg.kg^{-1}$60 mins prior to exercise	Maximal effort 21-km road races in hot humid conditions	**NO**	No effect of caffeine on race time, no change in the change in the concentrations of Na^{+}, K^{+}, glucose, lactate

Graham & Spriet (1991)	7 well trained competitive runners	9mg/kg 1 hour prior	2 trials running and 2 trials cycling to exhaustion at 85% VO_{2max}	**YES**	Endurance time increased during running from 49.2 ±7.2 mins to 71± 11 mins and cycling 39.2± 6.5 mins to 59.3 ±9.9 mins.Adrenalinwas increased prior to and during exercise. No effect on RER or plasma FFA
			Sustained High Intensity Activity		
O'Rourke *et al.* (2006)	The well-trained group 15 participants (age 32.2 ± 8.8 yrs) at least five years club-level competition experience. Recreational runners 15 participants (age 29.0 ± 5.7 y), history of playing team-sports	5mg.kg^{-1} 60 mins prior to exercise	5km time trial on running track	**YES**	Caffeine trial significantly improved running time by 1.1% in well-trained and 1% in recreational runners. Over all 27 of the 30 subjects recorded a faster time.
Bridge & Jones (2006)	8 male distance runners	3mg.kg^{-1} 60 mins prior to exercise	8km time trial on running track	**YES**	Caffeine trial resulted in a statistically significant 23.8s (1.2%) improvement in 8km running time, elevated blood lactate following the first 3 mins, higher heart rate, and a decreased RPE despite faster running speed
Bruce *et al.* (2000)	8 well trained male rowers	6 or 9 mg.kg^{-1} 45 mins prior to exercise	2000-m time trial rowing ergometer	**YES**	Low dose resulted in a 1.3% improvement in time to complete 200m, higher dose resulted in a 1% improvement. Blood lactate 22% higher & blood glucose 13% higher in caffeine trials. 50% increase in resting plasma FFA in low dose 100% increase in higher dose
Jackman *et al.* (1996)	3 women 11 men (aged 23.5±2) who participated in recreational endurance actives	6mg.kg^{-1} 60 mins prior to exercise	Cycle ergometer - 2mins at PO requiring Vo_{2max}/ 6 min rest/ repeated exercise/ 6 min rest/ repeated exercise until voluntary exhaustion	**YES**	Significant increase in endurance in caffeine trial when compared to placebo (4.12 + 0.36 and 4.93 + 0.60 min) Higher plasma adrenalin in the caffeine trial, no difference in muscle glycogen or blood lactate
Wiles *et al.* (1992)	18 male athletes (18-29 years)	3 g of caffeinated coffee60 mins prior to exercise	1500m running performance	**YES**	Mean time to complete decreased by 4.2 seconds in caffeine trial, the speed of the final min increased by 0.6 kmph

Table 1.4.1. - Example of literature showing how different amounts of caffeine supplementation, administration, and exercise protocols affect performance in endurance and sustained high intensity activity.

Author	Subjects	Caffeine Dose	Protocol	Performance Enhanced	Comments
			High Intensity Anaerobic Activity		
Carr *et al.* (2008)	10 male team sport players (aged 25±5)	6 mg.kg^{-1} 60 mins prior	5 sets of 6x20m sprints; sets 1, 3, and 5 had departure time of 25s, sets 2 and 4 60s.	**YES**	All sprints significantly faster in caffeine trial
Schneiker *et al.* (2006)	10 male amateur level team sport athletes	6 mg.kg^{-1} 60 mins prior	2 x 36 min cycling integrated with 18 x 4s sprints with 2 min recover	**YES**	Caffeine trial resulted in 8.5% improvement in total work in the first half and 7.6% greater in the second half. Sprint mean peak power increased by 7% in the first half and 6.6% in the second ($P<0.005$ in both cases)
Collomp *et al.* (2001)	6 healthy volunteers - non-specifically trained	5 mg.kg^{-1} 60 mins prior	Wingate test	**NO**	No significant change in anaerobic capacity, power and ability to maintain power. Significant increase in catecholamine and blood lactate
Stuart *et al.* (2005)	9 male rugby players	6 mg.kg^{-1} 70 mins prior	Specific rugby union test consisting of 7 circuits in 2 x 40 min half's with 10 min rest break. Measured sprint time (20 & 30m) drive power & accuracy of passing	**YES**	Sprint speeds improved, 0.5% through 2.9%; first-drive power, 5.0%; second-drive power, -1.2%; and passing accuracy, 9.6% (±6.1%). Attributed to reduction in fatigue and elevated arousal and concentration
Greer *et al.* (1998)	9 physically active males (aged 29.1±2.7) (Not accustomed to high intensity exercise)	6 mg.kg^{-1} 60 mins prior	30 second Wingate tests with four minute recovery	**NO**	No effect on power output (peak or average) performance declined in trial 3 and 4. Elevated adrenalin was demonstrated but this effect had ceased prior to testing.
			Muscle Strength and Fatigue of Muscle Strength		
Norager *et al.* (2005)	15 men and 15 women > 70 years – divided into group A (placebo then caffeine) and B (caffeine then placebo)	6 mg.kg 60 mins prior	The maximal voluntary isometric arm flexion strength was measured in the dominant arm using a strain gauge mounted on a dynamometer chair	**NO**	There was no significant increase in maximal muscle strength
Astorino *et al.* (2008)	22 resistance trained men	6 mg.kg 60 mins prior	1 rep max barbell bench press and leg press - followed by reps to failure at 60% 1 rep max	**NO**	No significant difference found in maximal muscle strength nor time to fatigue
Beck *et al.* (2008)	37 resistance trained men divided into caffeine (n=17) and placebo groups (n=20)	6 mg.kg 60 mins prior	1 rep max, muscle endurance (total volume of weight lifted at 80% 1 rep max) for free weight bench press and leg press	**YES**	Significant increase in bench press 1 rep max in caffeine group no significance in any other parameters measured

Table 1.4.2. – Example of the literature showing how caffeine supplementation affects different protocols of high intensity anaerobic activity and maximal muscle strength performance

Investigation of the literature in this area has highlighted many limitations to the previous human based studies conducted and thus explains some of the variability in results and conclusions gained. As caffeine can be found in abundance in the energy dense western diet it is difficult to fully control subject's blood plasma caffeine content prior to conducting the exercise protocol and in early studies this factor was not considered. Bruce *et al.* (2000) attempted to manage this variable by insisting on nutritional control of the participants. Restrictions were placed on caffeine 72 hours prior to testing and nutritional intake dictated 24 hours before each trial. It is widely considered that caffeine is an addictive stimulant (Fredholm *et al.* 1999), thus can the food diaries provided be satisfactory proof that cessation of caffeine ingestion occurred during the 72 hours before the trials? Also the problem of caffeine withdrawal from habitual consumers should be considered. Juliano and Griffiths (2004) reported some of the primary symptoms of caffeine withdrawal to be headache, fatigue, decreased energy/alertness, drowsiness and muscle pain/stiffness among others. Symptoms occur within 12-24 hours post withdrawal reaching peak intensity between 20-51 hours. The overall duration of the symptoms last for 2-9 days. Habitual users may display further non-respondent effects to caffeine consumption compared to subjects that are no habitual consumers.

Optimal prescription of caffeine use in relation to timing and dosage is a factor to be considered when examining human studies. It is not uncommon for subjects to be given an absolute dose of caffeine treatment rather than a dosage as a proportion of their body mass. Therefore in such studies blood caffeine concentration is not consistent throughout subjects resulting in misleading results due to a large variability in dosage causing variability in response. Caffeine is rapidly absorbed and is usually consumed an hour before the exercise protocol. In relation to this Graham (2001) reported that it was rare to find instances where plasma concentrations had been measured in subjects prior to execution of the exercise protocol thus discrepancy between participants was not distinguished. Ample evidence has been collected in human and animal studies highlighting that some tissues are able to adapt to long-term caffeine exposure resulting in a reduction of its effect. Thus posing the question could caffeine provide greater benefit in some athletes who have not been exposed? Evidence for this is apparent throughout

the literature; a prime example being a study conducted by Bell and Mcleellan (2002). Bell and Mcleellan (2002) divided their subject pool into users (consumption > 300 mg/d) and non-users (consumption < 50 mg/d) and concluded that the treatment led to increased time to exhaustion in both groups, however the effect lasted approximately 3 hours longer in the non-users.

A variation in the subject pool between studies is also evident. Commonly differences in age, sex and performance level exist with some studies using elite athletes (table 1.4.1; table 1.4.2.). This variability could go some way to explaining the variation in results and individual response level.

Some of these limitations outlined in human research are addressed in the animal model, all muscle preparations receive the same direct dosage of caffeine, and thus variation in digestion is eradicated. Furthermore, mice don't have a diet high in caffeine so potential habituation effects are also eliminated.

Adverse Effects of Caffeine Consumption

Further to the previously discussed effects of caffeine withdrawal, prolonged consumption of high dosage of caffeine can have adverse physiological effects on performance and health. A review by Smith *et al.* (2002) explored how high doses of caffeine can lead to increased anxiety in some individuals and impairment in fine motor control. Further to this Smith *et al.* (2002) displayed evidence for the common theory that caffeine will decrease one's ability to sleep. However, it was further suggested that humans are good at regulating their caffeine consumption and will not consume high doses at night.

Caffeine has also been recognised as having a diuretic action, Maughan and Griffin (2003) reviewed caffeine ingestion and fluid balance and concluded that caffeine-containing beverages result in excess fluid loss resulting in a poor hydration status. Large, acute doses of at least 250mg resulted in short term stimulation of urine output. The result of this is a decrease in plasma volume and electrolyte balance, which may decrease time to fatigue in prolonged exercise.

There is also evidence showing that caffeine will accelerate bone mineral density (BMD) loss. Harris and Dawson-Hughes (1994) concluded that daily consumption of high doses caffeine accelerated BMD loss

from the spine and total body in healthy postmenopausal women with calcium intakes below the recommended daily intake. Similar results were further confirmed by Rapuri *et al.* (2001). Controversial are the effects of high dose caffeine consumption on risk of coronary heart disease; the most recent literature has shown that caffeine does not increase the risk of Coronary Heart Disease (CHD) and death (Kawachi *et al.* 1994; Kleemola *et al.* 2000; Lopez-garcia *et al.* 2006).

Caffeine Mechanisms of Action - CNS

A review by Lorist and Tops (2003) explored the behavioural and performance response to caffeine ingestion and demonstrated an increase in response to stimuli, an elevated state of arousal and a decreased rate of perceived exertion with support from EEG data. In much of the literature caffeine is considered a psycho stimulant along with amphetamines and cocaine, as it results in similar behavioural responses. The most widely established behavioural effects of caffeine are on motor activation and arousal (Fredholm *et al.* 1999).

The most extensive evidence suggests that caffeine functions as an adenosine receptor antagonist (George, 2000). Due to the similar molecular structure as adenosine, caffeine will bind to adenosine receptors thus inhibiting the binding of adenosine and its functions (Kalmar & Cafarelli, 2004). Caffeine will act on the four cloned receptors to a varying extent however it is believed that at A_1 receptors highly expressed in the brain and A_{2a} receptors of large concentration in the striatum the effect is greatest (Ferre, 2008).

A_1 receptors are responsible for reducing neuron firing and result in vasodilatation of blood vessels allowing increased oxygen consumption during sleep. A decrease in muscle activity is achieved via a decrease in cAMP formation and inhibiting the function of voltage dependant calcium channels cumulatively important in the transfer of neural impulses (Fredholm *et al.* 1999). Reducing neuron firing will result in a lower peak force and slower time to activation, as the rate of Ca^{2+} efflux from the SR thus the intracellular concentration will be lower.

A_{2a} receptors are believed to play a key role in promoting a sleep like state; failure of this process via caffeine ingestion will result in an arousal enhancing effect (Ferre, 2008). Activation of A_{2a} receptors leads to decreased affinity of dopamine binding to D2 receptors. The binding of adenosine to A_{2a} receptors has been shown to promote the release of GABA, the primary inhibitory neurotransmitter. However, the binding of dopamine to D2 receptors promoted via caffeine has been shown to block the release of GABA. It has been established that a substantial increase in the concentration of caffeine increases the turnover of several neurotransmitters notably dopamine. Inhibition of adenosine receptors via caffeine will result in an increased transmission via dopamine at D2 receptors (Fredholm *et al.* 1999).

As a further consequence of caffeine treatment, the pituitary gland senses the increase in neuron firing and responds by releasing adrenalin, a caffeine-induced, increased plasma adrenalin level was concluded in studies by Jackman *et al.* (1996) and Graham *et al.* (1998). The net result of increased adrenalin is an increased heart rate, blood pressure and vasoconstriction of the blood vessels and arteries, and increased glycogenolysis. Adrenalin is also believed to promote enhanced oxidation of free-fatty acids thus promoting glycogen sparing, contributing to increased endurance performance (Davis & Green, 2009) however this occurrence was not concluded by Graham *et al.* (1998). Spriet *et al.* (1992) took muscle biopsies from subjects cycling at 80% VO2max to exhaustion and concluded decreased glycogenolysis by approximately 55% during the first 15 minutes of exercise following caffeine treatment. An increase in FFA's was also concluded throughout much of the literature (e.g. Essig *et al.* 1980: Bruce *et al*; 2000). Rush & Spriet (2001) further supported the mechanism of glycogen sparing concluding that caffeine at levels of moderate human consumption can inhibit and reduce the sensitivity of glycogen phosphoralase, therefore reducing the rate of glycogenolysis. Chesley *et al.* (1998) reported that muscle glycogen decreased by 28% following caffeine ingestion in half of the subject population, and was unaffected in the other half. However,

Chesley *et al.* (1998) further reported that caffeine significantly increased resting FFA concentration and adrenalin concentration twofold during exercise in all subjects. This, therefore, shows an increase in FFA via the mechanism induced by caffeine supplementation may not necessarily result in glycogen sparing. As with many of the mechanisms in this area, contradicting literature is also evident here. Graham (2001) questions the glycogen sparing theory as the manner promoting increased endurance capacity. Well documented is the fact that caffeine will elevate circulating catecholamines, conversely the increased mobilization of FFA's as a knock on effect has not been verified. More recent articles reviewed concluded that a decrease in Respiratory Exchange Ratio (RER), where a reduced value represents a greater fat metabolism for energy production, has not been demonstrated during prolonged activity (Graham *et al.* 2008; Turley *et al.* 2008; Ivy *et al.* 2009). A further confounding theory is a reported increase in blood lactate following the consumption of caffeine (Graham *et al.* 1998; Bell & Mclellan, 2002) that contradicts the glycogen-sparing hypothesis with carbohydrates being essential in this form of metabolism.

Caffeine Mechanisms of Action – The Effects of High Concentrations of Caffeine on Skeletal Muscle

The importance of *in vitro* studies centre on the isolation of a muscle tendon unit allowing its properties to be compared in caffeine and non-caffeine trials. As previously outlined, in human studies it is difficult to isolate factors that result in direct muscle performance improvement from muscle performance improvement resulting from central mechanisms. However an isolated muscle is externally stimulated and its metabolism controlled, thus it is possible to exclusively examine the skeletal muscle reaction to a caffeine dose. The direct treatment of muscle with caffeine eliminates individual differences in metabolism that occur between humans and ensures that all muscle preparations are subject to the same dose over identical periods. Additionally the animals from which these preparations are taken do not have a diet high in caffeine eliminating this variable that is difficult to control in human investigations.

Much of the evidence of the direct effects of caffeine as an ergogenic aid on muscle derive from the early *in vitro* studies such as Luttgau & Oetliker (1967) who tested millimolar caffeine treatment (supraphysiological for humans) on isolated semitendinosus and iliofibularis muscle in Swiss mountain frogs. The study concluded significant increases in twitch force following treatment with 6-10 mM caffeine and an increased sensitivity to caffeine following a drop in temperature from 20^{o}C to 1-3^{o}C.At high concentrations caffeine has even been shown to produce contracture without stimulation (Huddart, 1968).

The primary mechanism by which caffeine can promote greater force output in skeletal muscle is believed to be via interference with excitation contraction coupling (Davis & Green, 2009). It has been established that the specific mechanism of action lies with alteration in the intramuscular ion handling, primarily concerning the increased concentration of Ca^{2+} within the intracellular space (Magkos & Kavouras, 2005). The role of Ca^{2+} in promoting muscle activation has already been explained, however it is believed by increasing the intracellular concentration promotion of faster muscle activation and therefore a greater power output will occur. One of the earliest studies that investigated this theory was Weber & Herz (1968). They isolated SR from frogs and monitored Ca^{2+} release to varying caffeine concentrations. Caffeine caused an immediate release of Ca^{2+} in 11 of 12 preparations. It was established that a shift in the voltage dependant Ca^{2+} release mechanism to a more negative membrane potential resulted in this phenomenon. This mechanism was later confirmed by Endo *et al.* (1970) using skinned muscle preparations with SR left intact.

Allen *et al.* (1989) demonstrated that a 10mM caffeine treatment promoted an enhanced opening of the RyR2 channels of the SR resulting in a greater Ca^{2+} influx into the intracellular space. A later study by Allen & Westerblad (1995) provided additional evidence demonstrating that a caffeine treatment led to a decrease in the sensitivity of the SR Ca^{2+} pump and an increased SR Ca^{2+} permeability. Consequently the rate of Ca^{2+} efflux from the intracellular space will be significantly slower during caffeine treatment, resulting in a greater intracellular Ca^{2+} concentration at rest and during activation (Allen & Westerblad,

1995). Magkos and Kavouras (2005) further suggested that if Ca^{2+} is released from SR at a quicker rate then this will result in a quicker response of the Ca^{2+} induced Ca^{2+}response mechanism. Allen and Westerbald (1995) also recognised that caffeine caused an increase in myofibrillar Ca^{2+} sensitivity.

The overall effect of caffeine is a modified Ca^{2+} transient time resulting in an increase in basal and activated intracellular levels of Ca^{2+} in conjunction with an elevation in the plateau phase (Fryer & Neering, 1989). A combination of these factors will result in a decrease in the muscle's ability to restore the intracellular concentration back to pre-treatment resting levels thus making it harder for the cessation of muscle activation. Allen & Westerblad (1995) demonstrated a decreased rate of muscle relaxation in mouse flexor brevis muscle. Along with an increased Ca^{2+}concentration due to caffeine treatment, the intracellular caffeine concentration would have to fall further to achieve relaxation due to an increased Ca^{2+} sensitivity. Another possibility is that as a result of this increased sensitivity, it may take longer for Ca^{2+} to dissociate from troponin. Therefore it is likely that a decreased rate of muscle deactivation may be due to alterations in muscle Ca^{2+} transient time. During locomotion a decrease in time to relaxation may prove to be disadvantageous, as an active muscle undergoing lengthening will result in elevated passive force.

A further proposed mechanism as to how caffeine could modify excitation contraction coupling lies with increased stimulation of Na^+/K^+-ATPase activity (Davis & Green, 2009). The ability of a cell to maintain plasma K^+ homeostasis is essential in the maintenance of cell membrane potential. If this can occur more readily with greater efflux of Na^+ and influx of K^+ action potentials will be able to propagate at a faster rate. Therefore Ca^{2+} can be released at a faster rate in response to an oncoming stimulus.

Many *in vitro* studies have found that caffeine treatment elicits acute one off enhancement of force (e.g. Huddart 1968; Fryer & Neering, 1989; Allen & Westerblad, 1995; Germinario *et al.* 2004; James *et al.* 2004; James *et al.* 2005), however this benefit has not been shown to transfer to prolonging muscle power output with studies by Germinario *et al.* (2004), Reading *et al.* (2004), James *et al.* (2005) all concluding against an increase in time to fatigue. Germinario *et al.* (2004) concluded that caffeine

significantly reduced time to fatigue at maximal and sub maximal activation levels in mouse EDL and soleus muscle.

Effect of Physiological Doses of Caffeine *in vitro*

Graham (2001) provided an in-depth review of caffeine and exercise in relation to metabolism, endurance and performance effects along with possible underlying mechanisms. The initial significant point to be noted is that caffeine within human physiological concentrations is usually below 70 µmol/l with common blood plasma levels being 20 – 50 µmol/l. However, most *in vitro* investigations have used non-physiological caffeine concentrations of between 500 – 5000 µmol/l. Such studies have no physiological relevance to humans as blood caffeine levels exceeding 70µM are toxic, with levels exceeding 1mM often fatal (Fredholm *et al.* 1999). This lack of human relevance in relation to the caffeine concentration used suggests that conclusions drawn from such experiments to be of little value in relation to investigation of the drug as an ergogenic aid in humans

Many of the initial *in vitro* studies are also limited due to the physical environment that the preparations were tested in. James *et al.* (2004; 2005) were the first to mimic the physiologically relevant conditions of human muscle and used a caffeine dosage that was attainable in blood plasma. James *et al.* (2005) used mouse EDL (relatively fast muscle) to assess the effects of micromolar caffeine concentration on force production and power output in non-fatigued muscle over short or prolonged cyclical activity. Further to this the effect of caffeine on recovery from fatigue was also investigated. It is the methods used that provide the James *et al.* (2004; 2005) studies with their greatest validity. During testing the muscle was maintained in Krebs-solution at a temperature of 35^{o}C. The Krebs solution contains glucose as well as micromolar concentration of a number of other substances at a pH of 7.60 (reduced to pH 7.4-7.5 during the experiment as it is bubbled with 95% O_2, 5% CO_2) allowing simulation of the blood plasma that surrounds mammalian muscle *in vivo*. Principally, this solution was maintained at a temperature of 35^{o}C comparable to physiological temperature. Caffeine treatments of 70µM were added to the Krebs solution, representing a maximum level of caffeine relevant to blood plasma *in vivo* (Fredholm *et al.* 1999), making

this work conducted by James *et al.* (2005) unique for mammalian muscle. The work loop technique was implemented as a practice to incorporate the dynamic conditions of length, force and velocity that simulate the cyclical length changes that can occur in skeletal muscle *in vivo*. James *et al.* (2005) concluded that small but significant increases (2%) in mean net muscle power output occurred after caffeine treatment, attributed to increased force production during shortening. Further to this a marked performance difference between preparations was present. As demonstrated in the human research (Table 1.4.1 and 1.4.2), response to the caffeine treatment should therefore be viewed on an individual level highlighting a division of responders and non-responders. The caffeine induced potentiation of maximal power was not maintained throughout fatigue tests, thus 70μM caffeine treatments had no significant effect on delaying the onset of fatigue or enhancing recovery from fatigue. Further to this no enhancement in the recovery from fatigue with 70μM caffeine treatment was reported by James *et al.* (2004).

It has clearly been demonstrated that caffeine is an ergogenic aid. The 2% direct increase in muscle power output established by James *et al.* (2005) *in vitro* could cause measurable improvement in sporting performance, especially in sprint events where tenths of a second separate athletes. Constant throughout the *in vivo* and *in vitro* literature is a division of subjects into responders and non-responders (e.g. James, 2005; Skinner *et al.* 2009), such that for responder's high level caffeine consumption could provide an advantageous effect. In conjunction with this direct effect, caffeine could also cause CNS improvements and debatable beneficial substrate metabolism to further increase performance (Graham, 2001). Caffeine has always had a firmly established role as a stimulant increasing awareness and reaction time thus further cementing its function as a performance enhancer (Yacoubi *et al.* 2000).

Caffeine Concentration in the Present Research

As with previous research, the present work will use 70μM caffeine concentration to represent the human maximum physiologically attainable caffeine concentration in blood plasma (James *et al.* 2004; 2005; Rosser *et al.* 2009). Caffeine is almost completely (99%) absorbed from the gastrointestinal tract

and its hydrophobic nature allows free passage across all biological membranes allowing distribution to all bodily tissues (Magkos & Kavouras, 2005; Fredholm *et al.* 1999). However, the distribution of caffeine to these tissues is unlikely to be equal in magnitude across all tissues. Berg and Werner (1971) examined the tissue distribution of caffeine and its metabolites in a number of tissues from mice. The results initially indicate that the tissue concentration of caffeine is varied and does not equal that of the blood plasma. Importantly, Berg and Werner (1971) further demonstrated that the muscle caffeine concentration was relatively high after 30 and 60 minutes compared to other tissues measured, and that the concentration in blood plasma remained relatively constant over the 30 minutes measured. Results from Berg and Werner (1971) in part confirm earlier findings suggesting that caffeine distributes in tissue in proportion to tissue water. In light of this evidence, 70µM caffeine concentration is deemed the most appropriate maximum physiologically relevant concentration in the context of the present work, as tissue distribution is dependent on the concentration absorbed into the blood. In the present work skeletal muscle is isolated and placed in a flow through chamber with circulated oxygenated Krebs-Henseleit solution, which acts to mimic blood plasma. The caffeine in the Krebs-Henseleit solution is free to diffuse into the muscle as it would *in vivo*.

1.5. Changes in Skeletal Muscle Function with Age

Sarcopenia refers to a reduction in muscle mass, strength, and a slowing of contraction that is an inevitable occurrence with age (Williams *et al.* 2002). Sarcopenia affects mobility, increasing the risk of falls and fractures and reducing quality of life in the elderly. Despite the demonstrable benefits of training (specifically strength enhancing exercise) it is unfeasible to fully offset the age related decline in muscle performance and changes in body composition (Klitgaard *et al.* 1990). By taking measurements of muscle area, total fiber number, fiber size, proportion and distribution of fiber types of whole vastus lateralis in men aged between 15 and 83 years, Lexell *et al.* (1995) confirmed that muscle atrophy begins after 25 years of age and accelerates during the later stages of life. Losses in absolute muscle force production were attributed to a decrease in muscle cross-sectional area (CSA) derived from a reduction in fiber size or fiber number or a combination of these factors. Decreases in physical activity, alterations in hormone release, chronic disease, and malnutrition are factors that have been identified to contribute to the loss in muscle mass and strength (Baumgartner *et al.* 1999). The ageing effect is known to negatively impact on force, power and endurance parameters of muscle performance. A key point to note here is that an age related decline in muscle performance occurs considerably before what is typically determined 'aged', thus a greater understanding in the rate of decline of skeletal muscle function at various points beyond physical maturity is needed.

The Effect of Age on Muscle Strength & Power

A reduction in muscle strength and power is a commonly recognised consequence of ageing (Doherty, 2003; Deschenes, 2004). It is important to initially distinguish the differences between muscle strength and power. Muscle strength can be defined as the maximal amount of force exerted in a single attempt (Deschenes, 2004). Muscle power output is a product of load multiplied by velocity; this indicates that muscle power is the rate at which work can be produced (Macintosh, 2006).

Murray *et al.* (1980; 1985) reported that isometric strength of the knee flexor and extensor muscles reduced by an average of 55-65% and 56-78% in elderly men and women respectively. A loss in the capacity of aged muscle to produce isometric tension is also well supported by *in vitro* research using isolated mammalian muscle (Brooks & Faulkner, 1988; Zhang & Kelsen 1990). Muscle strength can be maintained fairly well up to middle age; however, the rate of decline increases more rapidly beyond this. Longitudinal studies such as Frontera *et al.* (2000) have shown 20-30% reductions in the isokinetic muscle strength of the knee and elbow extensors over a 12-year period in elderly men. An earlier 7 year follow up study Aniansson *et al.* (1986) examining quadriceps strength in elderly men demonstrated strength decreases of 10 - 20%.A reduction in muscle strength and power is evident in men and women beginning by age 40 (Metter *et al.* 1997). The decline in power has been shown to be significantly faster than strength (Metter *et al.* 1997). Skelton *et al.* (1994) examined muscle strength and power in 50 men and 50 women evenly distributed over the age range 65-89. A yearly overall 1-2% and 3% loss of isometric strength and leg extensor power respectively was estimated, however the rate of decline was significantly faster in men.

An age related depression in the force-velocity relationship and overall reduction in maximal unloaded shortening velocity (V_0) has also been firmly established which may contribute to the increasingly rapid loss of muscle power (Raj *et al.* 2010). The two constituents of muscle power are force multiplied by velocity, the recognised age related decline in muscle strength and V_{max} (the two constitutes of the power output equation) will result in a significant reduction in maximal muscle power output. Krivickas (2001) used slack testing to determine that V_0 differed with age and gender. The V_0 of type IIa fibers in elderly men was 83% of that of their younger counterparts; however $V_0$was unchanged in type I fibers. On the other hand there was no difference in V_0 in type IIa fibers in elderly women, however, V_0 was reduced to 93% in type I fibers. A decrease in soleus V_0 in aged rat soleus has also been confirmed by Thompson and Brown (1999) using the same method. This is supported by an *in vitro* reduction is muscle isometric activation and relaxation time (Edstrom & Larsson, 1987). Conversely the *in vitro* reduction of muscle power output (78% and 73% for mouse EDL and soleus respectively) demonstrated by Brooks and

Faulkner (1988) was not matched by any age related changes in the force-velocity relationship. It was, therefore, concluded that the reduction in power output is mainly drawn from a reduction in the muscle force generating capacity via a decline in the number of cross bridges or a reduced force development by individual cross bridges.

Although a reduction in muscle strength is common in concentric, eccentric and isometric measures, it is considered that the age related reduction in eccentric muscle strength is less pronounced (Deschenes, 2004). Poulin *et al.* (1992) and Porter *et al.* (1995) have both demonstrated a greater maintenance of eccentric muscle torques in the elderly compared to that seen in concentric muscle action. It is considered that this may be the result of slower contractile properties or increased connective tissue contributing to increased stiffness in the aged muscle (Doherty, 2003)

The Effect of Age on Muscle Endurance

Muscular endurance is the ability of the muscle to resist fatigue. The age related effect of muscle fatigue is equivocal with some research providing evidence for a decrease in muscle endurance whereas other studies show no difference, or an increase, in the muscle endurance with increasing age (Deschenes, 2004).

Using a maximal multistage incremental cycling test, Izquierdo *et al.* (2001) reported a significant reduction (19 % when related to body mass) in maximal workload in older subjects. This was related to significantly reduced heart rate and blood lactate concentration in the older subjects. Research by Lennarken *et al.* (1985) and Davies *et al.* (1986) postulate that increased age leads to a reduction in skeletal muscle endurance.

Pagala *et al.* (1998) examined fatigue in isolated muscle preparations in young and old rats using sub maximal isometric tetanic contractions. A significant increase in the resistance of soleus to fatigue was reported in the old mice. Interestingly, Pagala *et al.* (1998) also examined whole animal endurance performance and reported that running and swimming duration in the older mice was significantly

reduced. Therefore it was concluded that the increased fatigue that is attributed to old age is not a response to impairment of the muscle ability but due to central or extra muscular processes. *In vivo* research by Kent-Braun *et al.* (2002), in part, supports these findings, reporting a greater fatigue resistance during intermittent, sub maximal, isometric contractions in older adults. This effect was shown not to be significantly different between genders nor directly attributed to a reduction in neural activation during activity.

In contrast Lindstrom *et al.* (1997) examined the effects of increasing age on muscle fatigue by comparing maximal knee extensor strength using isokinetic dynamometry between 28 year old and 73 year old men and women. Despite a reduction in maximal voluntary contraction elderly subjects showed no difference in rate of fatigue. Other studies report an age related decline in muscle strength with no significant effect on muscular endurance (Backman *et al.* 1995; Bemben *et al.* 1996). The conflicting findings are likely to occur due to differences in experimental methods (i.e. the fatigue protocol and the point at which the muscle is deemed fatigued), muscle groups tested, the intensity of exercise, and differences between maximal voluntary contraction and electrical stimulation of muscle.

Mechanisms of Skeletal Muscle Ageing

Ageing will affect all the major physiological systems in the body in some way; therefore it should be considered that the degenerative process leading to sarcopenia arises from a combination of molecular and cellular factors. These factors are outlined in Fig 1.5.1 and the key mechanisms relating to the reduction in muscle mass and strength are discussed in this review.

Fig 1.5.1. - Overview of the interacting mechanisms that result is sarcopenia (from Ryan & Ohlendieck, 2004)

Changes in Excitation Contraction Coupling and Ca^{2+} handling

The rate of muscle activation and force production is primarily determined by excitation contraction coupling and the intramuscular Ca^{2+} transient time (Berchtold *et al.* 2000). An age related reduction in functionality of this process has been established which would greatly contribute to the loss of muscle function (Navarro *et al.* 2001).

Delbono *et al.* (1995) demonstrated a significant reduction in Ca^{2+} release in fast fibers of human quadriceps, attributed to DHPR-Ryanodine Receptor uncoupling. This conclusion was also confirmed by Renganathan *et al.* (1997) who demonstrated an age related reduction in RYR1 uncoupled to DHPR in rat EDL and soleus muscle. A reduction in the voltage gated SR Ca^{2+} release mechanism will result in a decreased Ca^{2+} availability for the contractile proteins and consequently a reduction in contractile force. In conjunction with this an age related decrease in the SR function of chemically skinned fast twitch

muscle of the rat was reported by Larsson & Salviati (1989). The reduction in SR Ca^{2+} concentration and Ca^{2+} pump activity were not transferable to slow twitch fibers. Evidence by Hunter *et al.* (1999) supports these finding in humans by reporting a significant reduction in SR Ca^{2+} uptake and Ca^{2+} -ATPase activity in aged Vastus Lateralis of elderly women. Narayanan *et al.* (1996) concluded a reduction in SR Ca^{2+} pump function in rat soleus that was not as pronounced in faster gastrocnemius muscle. Supplementary to this the results of Narayanan *et al.* (1996) failed to show any significant difference in the quantity of Ca^{2+}-ATPase, ryanodine receptor or calsequestrin (Ca^{2+} binding protein) in the aged population. No age related reduction in SR calsequestrin has been firmly demonstrated in both skeletal and smooth muscle (Klitgaard *et al.* 1989; Xu & Narayanan, 1998). Besides significantly affecting muscle force production these previously described mechanisms are likely to contribute to the age related increase in muscle activation and relaxation times (Hunter *et al.* 1999: Brooks & Faulkner, 1988).Evidence by Ray *et al.* (1995) and Saborido *et al.* (1995) further suggests that nerve stimulation and muscle activity can significantly affect muscular composition of dihydropyridine and ryanodine receptors. The reduction in physical activity that occurs during ageing is likely to negatively affect muscle receptor composition and further amplify the ageing effect via the previously described mechanisms.

In an attempt to offset this reduction in excitation contraction coupling, and more importantly the reduction of SR Ca^{2+} release, Hill *et al.* (1996) demonstrated that the contractile proteins of aged rat EDL and soleus had an increased Ca^{2+}sensitivity. In conjunction with this a greater resting concentration of intracellular Ca^{2+} has been demonstrated in aged rat EDL and, to a smaller degree, soleus muscle (Fraysse *et al.* 2006). Fraysse *et al.* (2006) hypothesised that this increase in homeostatic Ca^{2+}concentration will contribute to age induced muscle weakness via changes in myosin heavy chain isoform and increased activity of calcium dependant calpain proteolytic enzymes. Interestingly the treatment of aged rats with growth hormone restored homeostatic Ca^{2+} concentration close to mature levels.

Denervation

Stalber and Fawcett (1982) used EMG to report a significant decrease in motor unit number in small muscles of the hand and foot after the age of 60; this was partly counteracted with an increase in motor unit size. The common cycle of denervation - renervation is diminished in aged muscle and a net denervation results in a loss of motor units and innervated fibers (Carlson, 2004; Deschenes, 2004). A progressive denervation of muscle fibers and a loss of fast motor units have been identified as the primary mechanisms for the deterioration of muscle function with age (Einsiedel & Luff, 1992; Wineinger *et al.* 1995; Kadhiresan *et al.* 1996, Carlson, 2004). It is considered that ageing greatly reduces the number of motor axons supplying muscles. The loss of alpha-motorneurons is greater at type II muscle fibers, therefore these fibers cannot be activated and thus degeneration occurs (Carlson, 2004). Lexell (1995) confirmed this notion via comparisons of muscle biopsies between younger and older individuals revealing that type II fibers were smaller in older individuals while type I fibers were less affected. Kadhiresan *et al.* (1996) displayed evidence of motor unit remodelling in aged rat gastrocnemius muscle. The number of slow motor units remained constant; however, the number of fibers per motor unit increased 3 fold. Conversely, the number of fast fatigable motor units decreased by 34% in conjunction with an 86% reduction in number of fibers per motor unit. There was only a limited motor unit remodelling in fast fatigue resistant units.

Further considerations should also be given to the age related changes in neuromuscular junction morphology, particularly changes in acetylcholine receptor density (Courtney & Steinbach, 1981; Andonian & Fahim, 1987; Li *et al.* 2011).

Muscle Mass & Fiber Type Composition

The age induced reduction in muscle mass is primarily a consequence of a reduction in fiber number or a decrease in cross sectional area (Deschenes, 2004; Navarro *et al.* 2001). In humans, muscle mass peaks at 24 years, with only a small (10%) decrease by the 5th decade with a further, more rapid, 30% reduction by

the 80th year (Deschenes, 2004).Navarro *et al.* (2001) suggested that a reduction in motor unit number and size, decreased protein synthesis, disuse atrophy mechanisms, and alterations in hormone production made up a number of interacting factors that resulted in muscle wasting.

A reduction in the synthesis of myofibrillar proteins, with age, would explain the age related loss in muscle function and mass (Navarro *et al.* 2001). Contractile fore is dependent on the quality and quantity of the contractile proteins that are continually remodelled via protein catabolism and synthesis (Balagopal *et al.* 1997). Balagopal *et al.* (1997) reported a progressive age related decline in myosin heavy chain synthesis; this is likely to reduce the muscle's ability to remodel contractile protein and is expected to contribute to the age induced loss of muscle mass and strength. Welle *et al.* (1993) reported a one third reduction in quadriceps strength between young and old subjects. This was consistent with a 21% reduction in muscle mass and was further supported by evidence highlighting a reduction in myofibrillar protein synthesis between young and old populations. In conjunction with a loss of oxidative capacity an age related reduction in mitochondrial protein synthesis may also affect muscle mass (Rooyackers *et al.* 1996).

Detraining or the loss of conditioning is primarily associated with highly trained individuals that reduce or cease to adhere to their training programme. The reduction in training stimulus results in disuse atrophy that should also be considered as a mechanism in the aged population. Frontera *et al.* (2000) reported isokinetic muscle strength reductions ranging from 20-30%, after analysing muscular performance in elderly men (65.4 ± 4.2 yr) over a 12-year longitudinal study. Fiber cross sectional area had decreased by up to 16.1%, and although not the only highlighted mechanism, a significant reduction in the amount of physical activity was reported. Further support for the disuse atrophy mechanism can be seen in the literature that demonstrates training in the elderly can significantly increase muscle force and muscle CSA (Roman *et al.* 1993; Fiatauone *et al.* 1990). In essence those that participate in a greater amount of physical activity have improved muscular function.

It is well established that different fiber types can be distinguished, in each muscle, dependant on functional and structural properties. Alnaqeeb and Goldspink (1986) reported that the loss of muscle mass should be attributed to a reduction in fiber size and number. A key aspect of ageing phenotype appears to be a shift towards a more oxidative metabolism as the reduction in the CSA of type IIx fibers appears to be more pronounced (Aniansson *et al.* 1986; Coggan *et al.* 1991). Alnaqeeb and Goldspink (1986) showed significant reductions in the aged rat soleus FOG fiber diameter by up to 35.8% with only a marginal reduction in SO fiber diameter. EDL had a significant 22.7% reduction in the CSA of FG fibers, whereas the CSA of FOG fibers increased by 12.4% in conjunction with a relatively small increase in SO CSA. Despite early work suggesting that muscle fiber number decreases more rapidly in type II fibers it is now widely established that muscle fiber number will decrease equally across fast and slow fiber types(Deschenes, 2004). Lexell *et al.* (1988) reported a loss of muscle fibers in aged human vastus lateralis muscle, but more significantly they reported that this reduction did not differ between fiber types. Lexell and Downham (1991) further suggested that the random dispersal of fiber types seen in young muscle decreases with old age and that there is evidence of grouping of particular fiber types into clusters demonstrating muscle regeneration. The overall conclusion here is that slower muscle fiber types represent a greater proportion of the CSA of the muscle in aged muscles.

Increased Connective Tissue and Intramuscular Fat

Fibrosis causes an increase in skeletal muscle connective tissue; this reduces the flexibility of the muscle and can restrict movement and circulation (Martini *et al.* 2000). Alnaqeeb *et al.* (1984) examined connective tissue in aged rat EDL and soleus muscle, and found that there was an age related increase in muscle stiffness (length-passive tension) attributed to an increased thickness of the endomysium and perymysium, and greater total muscle collagen.

A further point to consider is the age related increase in intramuscular fat mass. Kent-Braun *et al.* (2000) reported an increase in intramuscular fat from 6% in young subjects to 15% in elderly subjects irrespective of gender. Kent-Braun *et al.* (2000) further reported the importance of physical activity in the elderly in

order to reduce intramuscular fat content. As a result of an increase in connective tissue and intramuscular fat content the aged muscle has a greater proportion of non-contractile mass, so is likely to generate a lower force per muscle cross-sectional area.

Hormonal Factors that Affect Skeletal Muscle Ageing

It is important to consider the effects of circulating hormones on aged muscle, many of which are involved in the anabolic process of muscle building. An age related reduction in such hormones could significantly contribute to sarcopenia (Deschenes, 2004).

Testosterone

Testosterone secreted in the testes of males is primarily a male sex hormone promoting androgenic and, more importantly in this instance, anabolic properties that cause skeletal muscle growth (Hebst and Bhasin, 2004). Gray *et al.* (1991) conducted a meta-analysis on the association between testosterone and male ageing and showed that, in research using only healthy subjects, a gradual age related decline in testosterone secretion occurred. A reduction in testosterone has been associated with a loss of muscle mass and strength, this may further be associated with impairment of the biological action of androgens in target cells (Gooren *et al.* 1998).

The underlying mechanism for reduced testosterone is not fully understood, however it is likely that there are changes in the different levels of the hypothalamo–pituitary–testicular axis (Gooren *et al.* 1998; Deschenes, 2004). It has been shown that testosterone producing Leydig cells have a reduced responsiveness to luteinising hormone (LH) that is secreted by the pituitary gland to increase blood testosterone concentration (Kaufman *et al.* 1990). It is further proposed that there is a reduction in gonadotrophin releasing hormone, from the hypothalamus, which regulates the release of LH from the pituitary gland (Vermeulen *et al.* 1989: Kaufman *et al.* 1990). Further to this an age related increase in the binding of testosterone to high affinity sex hormone binding globulin (SHBG) occurs, rendering it to be

biologically unavailable (Gooren *et al.* 1998). Gooren *et al's* (1998) review further suggested evidence of a reduction in testicular blood flow that would contribute to these aforementioned effects.

The use of testosterone replacement treatment for its anabolic effects on skeletal muscle has been widely studied. Bhasin *et al.* (1996) reported that a supraphysiological dose of testosterone significantly increased fat free mass, muscle size, and strength in healthy young men and that these effects were further pronounced when combined with strength training. It has been firmly established that testosterone replacement treatment can be used to reduce the effects of sarcopenia. Urban *et al.* (1995), Ferrando *et al.* (2001), and Hebst and Bhasin (2004) are just a selection of studies that have indicated that increasing testosterone levels in elderly men significantly increases mRNA concentrations of IGF-I and that this in turn resulted in increased skeletal muscle protein and strength.

Growth Hormone (GH) and Insulin-Like Growth Factor I (IGF-I)

As well as acting on a number of target tissues GH, secreted from the anterior pituitary gland in response to hypothalamic signals, stimulates the production of IGF-I in the liver (Navarro *et al.* 2001; Deschenes, 2004). IGF-1 has growth-promoting effects on almost all the cells in the body, particularly in skeletal muscle. As with testosterone, an age related decrease in free GH has been demonstrated (Rudman *et al.* 1981; Veldhuis *et al.* 1997; Vermeulen, 2002). A reduction in GH secretion, and the supplementary decrease in IGF-I, has been coupled with a decrease in muscle mass and strength, and a reduction in muscle protein synthesis (Welle *et al.* 1998).

In light of this, research has examined the effect of increasing GH on skeletal muscle performance. In a study by Papadakis *et al.* (1996) GH was given to elderly men with low IGF-I levels, over a 6-month period, then body composition and functional ability was assessed. The GH group displayed a 13.1% decrease in fat free mass but no significant effect on knee or handgrip strength, or systemic endurance. Yarasheski *et al.* (1995) investigated the interaction between GH secretion and resistance training in elderly men. The group that received GH treatment demonstrated a significant increase in fat free mass and whole body

protein synthesis; however this was not associated with improved muscle strength compared to controls. The majority of evidence tends to complement these trends suggesting that GH therapy is not effective in treating sarcopenia. Potential side effects and serious adverse health effects have also been suggested to occur with excessive GH administration (Yarasheski *et al.* 1995; Navarro *et al.* 2001).

Satellite Cells

Myosatellite cells are located within the basement membrane and the sarcolemma of muscle fibers (Mauro *et al.* 1961). These structurally undifferentiated cells are responsible for growth of muscle, undergoing proliferation and their nuclei fusing to existing muscle fibers to form new ones. Snow (1977) reported a 4.6% reduction in satellite cells in aged rat soleus muscle. A decrease in satellite cell number is a common response to ageing (Deschenes, 2004).

Mitochondrial Theory of Ageing

In skeletal muscle oxidative phosphorylation is the primary source of ATP regeneration. The mitochondria are the 'power house' of this process and as many other processes within muscle rely on ATP any reduction that may occur with ageing would significantly impede muscle function (Deschenes, 2004). Mitochondria are also the major site of formation of reactive oxygen species (ROS) and free radicals, that are responsible for cell oxidative damage of DNA, RNA, lipid and protein (Wei & Lee, 2002). Research by Short *et al.* (2005) supports the theory of DNA oxidative damage and reduced mtDNA with ageing, that is related to a loss of mitochondrial function in human skeletal muscle. ROS are formed via a leak of electrons from the electron transport chain, that then combine with oxygen to form superoxide anions ($O_2^{-}\cdot$); these then proceed to form ROS (Turrens, 2003). A significant build-up of ROS can largely be prevented by various antioxidant systems (Turrens, 2003).

As mitochondria function deteriorates with age it has been considered that this results in an increased leakage of electrons from the electron transport chain and increased formation of ROS. The production of antioxidants also significantly decreases with age exacerbating ROS accumulation (Wei & Lee, 2002).The

mitochondrial theory of ageing suggests that oxidative damage to mitochondria leads to a further production of ROS which develops into an escalating oxidative damage cycle, which over time causes mitochondrial dysfunction (Van Remmen & Richardson, 2001). It has been established that an accumulation of dysfunctional mitochondria occurs in ageing cells. de Grey (1997) proposed the 'survival of the slowest' hypothesis as a potential mechanism of this occurrence. Via this hypothesis it is thought that mitochondria selected for lysosomal degradation are those with high levels of membrane oxidative damage. The dysfunctional mitochondrial have reduced activity and are therefore inflicting reduced oxidative damage thus they are degraded at a slower rate. The development of this process over time results in an accumulation of dysfunctional mitochondria (Van Remmen & Richardson, 2001). A reduced turnover in mitochondria related to a reduction in mitochondrial protein synthesis will contribute to a decreased number of mitochondria with age (Rooyackers *et al.* 1996).

It has been further suggested that an increased oxidative stress can result in an amplified occurrence of cell apotosis via activation of the mitochondrial membrane permeability transition. This increased permeability draws in more water causing the mitochondria to swell and possibly leading to rupture of the outer membrane releasing cytochrome c, which can in turn lead to a cascade of reactions resulting in cell apotosis (Van Remmen & Richardson, 2001).

Besides significantly limiting skeletal muscle endurance capacity, the resultant decrease in ATP regeneration via oxidative phosphorylation, that results from reduced mitochondrial function, could also be a limiting mechanism for the discussed reduction in protein synthesis which requires large amounts of energy (Deschenes, 2004).

The Circulatory System

Reductions in cardiovascular performance cause a decrease in blood flow to the muscle at rest and at exercise (Martini *et al.* 2000). However, research has suggested that skeletal muscle capillary density is well maintained in the elderly (Chilibeck, 1997). *In vitro* research by Kano *et al.* (2002) used rat soleus and

plantaris muscle to also show the maintenance of skeletal muscle capillary supply in elderly muscle. Further to this the quality of the capillaries was also maintained, with the luminal size of each capillary being sustained with advancing age. Moller and Sylven (1981) reported slight increases in muscle myoglobin content in the aged population providing a further indication of the bodily desire to preserve skeletal muscle oxidative capacity.

Reducing the Effects of Ageing via Physical Activity

Ageing is related to a cycle of sarcopenia, whereby the age related reduction in muscle function results in muscle disuse and consequential disuse atrophy mechanisms and thus further sarcopenia. The elderly are encouraged to train within their physical limits in order to enhance their skeletal muscle function and in turn improves health and quality of life. The effect of a progressive training regime are well documented in mature adults, however, is it possible to offset the skeletal muscle ageing response via training and to what extent can this be achieved?

Endurance Training

Long-term endurance training in the elderly is associated with significant improvements in VO_{2max} of up to 20-30% (Seals *et al.* 1984; Hegberg *et al.* 1989; Coggan *et al.* 1992). The research of Coggan *et al.* (1992) exemplified the findings of the majority of literature within this area. They reported a 23% increase in VO_{2max} after 9-12 months aerobic training. In conjunction with this there was found to be a 9.1% increase in type IIa fiber CSA, an 8.1% decrease in type IIb fibers, CSA of type I fibers increased by 12% and type IIa by 10%. A supplementary significant increase in capillary fiber ratio and activity of mitochondrial enzymes associated with aerobic metabolism were further reported. Menshikova *et al.* (2006) demonstrated an increased skeletal muscle mitochondrial content after long-term endurance training in elderly men and women. These adaptations provide evidence for an increase in aerobic metabolism. It is considered that an increased capillary density is the most important adaptation, leading to an increase in muscle oxygen delivery (Kirkenall & Garrett, 1998).

A key question with regard to the decline in exercise performance with ageing is to what extent is this true effect of ageing and how much is it due to a reduction in the level of physical activity? As there are no studies that monitor a maintained training program over such a prolonged time-course (i.e. maturity to aged) it is hard to fully answer this question. However some light can be shed by looking at a few longitudinal studies that have been conducted assessing the effect on endurance capacity. Rogers *et al.* (1990) looked at the effects of VO_{2max} between elderly master endurance athletes and sedentary controls. Master athletes had been training from 10 years prior to the study and continued to train during the 8 year follow up study. Master athletes' VO_{2max} declined by 5.5% per decade compared to a 12% decline per decade in the sedentary controls. Sedentary subjects' HR decreased by 8 beats per minute which was not concurrent in the master athletes. Pollock *et al.* (1987) also assessed the effects of a maintained training program on VO_{2max} of elderly master track athletes over a 10-year period. Those that became non-competitive over this time had a significant reduction in VO_{2max}, whereas those that remained competitive had no significant change in VO_{2max}after 10 years. However, both subject groups displayed a 7 beats per minute reduction in maximal HR. The results indicate that long-term training is efficient at delaying the ageing effect on physiological systems. Without knowing the similar fitness parameters of the subjects in these studies from maturity (i.e. 20-30 years of age) up to old age it is still difficult to make an accurate conclusion. However, from what we do know it is fairly evident that a reduction in physical activity works as an accelerator to the loss of physiological function. The first point to note is that in the research by Pollock *et al.* (1987) there was no significant improvement in VO_{2max}, which you would expect in a progressive training program in younger adults. Further to this, the evidence presented shows the benefit of exercise in the elderly, but even longitudinal studies have failed to show that it is possible for elderly subjects to train their fitness parameters back to mature levels.

Resistance Training

Resistance training is employed as a method of improving a muscle's ability to produce force. This should be an important consideration for the elderly in an effort to reduce the risk of falls and resultant fractures

and as a method of increasing the functional aspect of life (Kirkendall and Garrett, 1998). Evidence suggests that a progressive program of high-intensity resistance training can result in substantial improvements in skeletal muscle force. Roman *et al.* (1993) indicated a significant increase in elbow flexor strength after 12 weeks heavy-resistance training in elderly males. There was a 41% increase in the amount of work achieved over 25 reps which was concurrent with a 13.9% and 22.6% increase in CSA of the biceps brachii and the brachialis respectively, and a 37.2% hypertrophy of type II muscle fibers. Resistance training has also been proven to be beneficial for people at the very extremities of human ageing. Fiatauone *et al.* (1990) demonstrated that 8 weeks high-intensity resistance training increased quadriceps muscle strength by 174% and muscle area by 9% in frail residents of nursing homes with an average age of 90 years.

The mechanisms of this increase in muscle strength will be the same as those that occur during resistance training in young adults. Besides the already reported increase in muscle CSA and changes in fiber type composition, Melov *et al.* (2007) showed that 6 months resistance exercise significantly increased muscular strength such that elderly subjects improved from 59% weaker than younger subjects to 38% weaker. This enhancement was partially attributed to the measured increase in mitochondrial function and, as previously suggested, partial reversal of the effects of the mitochondrial theory of ageing. Hunter *et al.* (1999) showed that high resistance training significantly increased the SR Ca^{2+} uptake and SR Ca^{2+} activity in vastus lateralis of elderly women. However this increase in SR Ca^{2+} uptake did not significantly improve muscle relaxation time and it was therefore concluded that Ca^{2+} uptake was not the rate-limiting mechanism for the slowing of relaxation time in aged muscle. The hypertrophic mechanism by which muscle responds to progressive training stimuli is likely to be limited in the elderly due to a reduced pool of satellite cells and the decrease in release of growth promoting hormones (Gray *et al.* 1991; Welle *et al.* 1998; Deschenes, 2004). Kraemer *et al.* (1999) demonstrated that, as with younger individuals, elderly men respond to resistance exercise with increased release of anabolic hormones. Despite this increase, total growth promoting hormone level will still be significantly lower compared to younger adults.

Long-term resistance exercise is sufficient at increasing muscle strength but not back to the levels achieved in young adults. McCartney *et al.* (1995) investigated the effects of 42 weeks of progressive weight training on dynamic muscle strength in the elderly. A mean increase of up to 65% in one rep max performance was reported independent of gender, yet 47% of this increase had occurred after 12 weeks. The study also reported an increase in muscle CSA. Further evidence by Pyka *et al.* (1994) showed a plateau in the gains in strength after 3 months in elderly subjects undertaking resistance exercise. These findings support the earlier hypothesis that ageing is an inevitable effect and that a reduction in physical activity works as a substantial accelerator of this process.

Ergogenic Effects of Caffeine in the Aged

With what is presented in the literature regarding the age associated reduction in skeletal muscle performance and the likely mechanisms, particularly alterations in fiber type composition and changes in Ca^{2+} handling, the question now posed is; does ageing effect the ergogenic properties of caffeine when directly applied at the skeletal muscle?

It is believed that in the elderly caffeine is metabolised in the same way as in young adults. However, due to changes in body composition a dose of caffeine consumed in the elderly will result in a higher plasma concentration. Caffeine is only distributed through lean body mass and ageing is associated with an increased adipose tissue to lean body mass ratio (Massey, 1998). Ageing affects all major physiological systems and little research has been conducted to investigate how the effect of caffeine supplementation changes with declining muscular function in aged populations.

Rees *et al.* (1999) reported significant reductions in psychomotor and cognitive performance with age; however, significant improvements were established after caffeine treatment which tended to be greater in the older subjects. The study reported that caffeine treatment in the elderly was efficient at offsetting the deficit in psychomotor and cognitive performance, in some tests, when compared to younger participants. As with young adults (Pincomb *et al.* 1985), Conard *et al.* (1982) reported significant

increases in blood pressure but no concurrent positive inotropic on the heart with caffeine treatment in elderly men.

A study by Norager *et al.* (2005) is the only study with any substance to assess the effects of caffeine supplementation on exercise performance in the elderly. They reported significant increases in cycling endurance (25%) and arm flexion endurance (54%), coupled with a reduction in RPE after 6mg/kg caffeine consumption in men and women aged over 70. No significant difference was found in muscle strength, walking speed and reaction time. Norager *et al.* (2005) noted that the increase in arm flexion was markedly higher than that seen in an earlier study on younger individuals.

The effect of caffeine on exercise performance of the aged is a pertinent area with the growing number of physically active elderly citizens and those completing rehabilitation programs. The research planned for study 4 and 5 will be the first to provide an insight into the direct effects of physiological concentration of caffeine on aged skeletal muscle. As we have established the muscle physiology is markedly different in the elderly and we will be the first to asses if the ergogenic is still applicable for the elderly and to what extent the changes through ageing.

Assessment of Skeletal Muscle Ageing

Although muscle atrophy and a loss of strength are generally accepted as an inevitable concomitant of ageing, research is limited as to whether skeletal muscles used for different functions age at the same rate. This study aims to use isolated mouse EDL, as an example of a fast, voluntary activated, peripheral locomotor muscle, compared directly against diaphragm, a mixed fiber type, primarily involuntary innervated, core muscle used in respiration, to investigate this hypothesis.

Voluntary Innervated Locomotory Muscles (EDL)

Brooks and Faulkner (1988) compared the contractile properties of skeletal muscle from young, adult and aged mice. They reported a significant reduction in tetanic force to 78% and 73% for EDL and soleus respectively. Further to this significant increase in activation and relaxation times were reported for the

soleus muscle only. Edstrom and Larsson (1987) further reported significant increases in twitch contraction and relaxation times in single motor units of isolated rat soleus with increasing age.

Gonzalez *et al.* (2000) investigated the isometric force of single intact fibers from elderly mouse EDL and soleus muscle. The study concluded a significant reduction in normalised force for both EDL and soleus that was more greatly pronounced in the predominantly fast twitch EDL. Later work by Gonzalez and Delbono (2001) investigated the relationship between age and isometric endurance, again using repetitive stimulation of single intact fibers from mouse EDL and soleus. The study concluded that age did not significantly affect the endurance of EDL and soleus muscle fibers irrespective of stimulation paradigm. Using similar methods Chan and Head (2010) further reported an age related reduction in the force generating capacity, an increase in twitch activation time, and a reduced fatigability in EDL of both male and female rats. Interestingly the aged muscle preparations showed no significant difference in muscle mass for female rats and an increase in EDL mass in male rats. This therefore indicates that the ageing response occurs before the establishment of muscle atrophy.

As with the majority of the literature examining sarcopenia, discrepancies in results can be largely attributed to differences between subject groups used and what is deemed as aged. Also reports of fatigue are subjective to what the authors deem as a substantial drop in muscle performance; particularly during *in vitro* research the idea of muscle fatigue has not been accurately defined.

Primarily Involuntary Innervated Respiratory Muscles (Diaphragm)

Dilation of the alveoli, decreased surface area for gas exchange, and a reduction in the static and elastic recoil of the lung are a sample of the mechanisms that increase the work of breathing in the elderly (Janssens, 2001). In a human model Poley *et al.* (1997) measured transdiaphragmatic pressure during a maximal sniff test in order to test the contractile properties of elderly human diaphragm. Poley *et al.* (1997) concluded a small (13%) but significant reduction in diaphragm strength. This is supported by a reduction in maximal isometric tension reported by Zhang and Kelsen (1990) that used isolated strips of

diaphragm comparing ageing golden hamsters. The importance of physical activity has been shown to significantly enhance respiratory muscle strength in the elderly. Summerhill *et al.* (2007) compared inspiratory and expiratory pressures and diaphragmatic thickness in subjects over 65 that were physically active against those who were inactive. The diaphragm thickness and inspiratory and expiratory pressures were greater in the active group.

As previously mentioned, physical inactivity in relation to ageing is a major contributor to muscle degeneration. This is likely to occur in soleus and EDL as these muscles are innervated voluntarily for whole body locomotion. On the other hand breathing occurs at a far more maintained rate with ageing and the diaphragm will be innervated to a greater extent. Therefore the level of decrement in muscle force production may be dependent on the muscle's physiological function.

2. Aims and Hypotheses of the Research

The rational for each study is discussed briefly in the general introduction and is explained in much greater depth in the introduction of each corresponding chapter.

Study 1 (Chapter 4)-**Effect of Physiological Concentrations of Caffeine on the Power Output of Maximally and Sub Maximally Stimulated Mouse EDL (Fast) and Soleus (Slow) Muscle**

Aims

To answer the following questions:

1. Are the effects of physiological concentrations of caffeine (70μM) different between muscles of predominantly different muscle fiber types (i.e. relatively fast EDL muscle versus relatively slow soleus muscle)?
2. Is the magnitude of the caffeine response increased if the muscle is stimulated sub maximally rather than maximally?
3. Does the ergogenic benefit of caffeine change over a range of physiological concentrations (35-140 μM)?

Hypotheses

1. Both mouse EDL and soleus muscle will demonstrate a direct force potentiation in response to 70μM caffeine treatment.
2. The effect of caffeine will be greater in muscles with a predominantly slow fiber type composition (e.g. soleus muscle).
3. 70μM caffeine treatment will induce a greater force potentiating effect in muscle stimulated at a sub maximal intensity when compared with muscle stimulated at maximal intensity.
4. There will be no dose dependant effect of caffeine treatment when used over a physiological concentration range.

5. Regardless of concentration, the direct response of skeletal muscle to caffeine will be subject to individual variation between preparations. Furthermore there is likely to be subgroups of responders and non-responders to the caffeine treatment.

Study 2 (Chapter 5)-**Does a Physiological Concentration of Caffeine (70µM) Affect Endurance In Maximally or Sub Maximally Stimulated Mouse Soleus (Slow) Muscle?**

Aims

To answer the following questions:

1. Does the treatment of mouse soleus muscle (relatively slow fiber type) with 70µM caffeine improve muscle endurance?
2. Are the caffeine-induced effects different when compared to previously studied mouse EDL muscle (James *et al.* 2005)?
3. Does the effect of caffeine on muscle fatigue resistance differ between maximal and sub maximal stimulation?

Hypotheses

1. Treatment of mouse soleus muscle with 70µM caffeine will significantly enhance muscle endurance. The effect will be different to that shown in EDL muscle where muscle endurance was unchanged following caffeine treatment (James *et al.* 2005).
2. The caffeine-induced enhancement of endurance will be significantly greater in sub maximally stimulated muscle compared to maximally stimulated muscle.

Study 3 (Chapter 6)- **Does a Physiological Concentration of Taurine Increase Acute Muscle Power Output, Time to Fatigue and Recovery in Mouse Soleus (Slow) Muscle With or Without the Presence of Caffeine?**

Aims

To answer the following questions:

1. Does the direct treatment of mouse soleus muscle with physiologically relevant concentrations of taurine significantly enhance maximal muscle force, endurance or the ability of the muscle to recover from fatigue?
2. Does taurine interact with physiological concentrations of caffeine to significantly enhance the ergogenic effects of caffeine?
3. Will the direct treatment of mouse soleus with physiologically relevant concentrations of caffeine significantly enhance the ability of the muscle to recover from fatigue? A comparison of the effect of caffeine on the recovery of EDL in an earlier study by James *et al.* (2004).

Hypotheses

1. Treatment of isolated muscle with physiological concentrations of taurine will not significantly improve maximal muscle power output, endurance, or the ability of the muscle to recover from fatigue.
2. Taurine when combined with 70µM caffeine will not significantly potentiate the effects of 70µM caffeine alone.
3. In contrast to James *et al.* (2004) where mouse EDL muscle was used, the direct treatment of soleus muscle with physiologically relevant concentrations of caffeine may provide a small enhancement in the recovery of muscle power output following fatigue.

Study 4 (Chapter 7)-**Is the Age Related Decline in Skeletal Muscle Function Muscle Specific? The Effect of Ageing on isolated Locomotory (EDL) and Respiratory (diaphragm) Skeletal Muscle Performance**

Aims

1. To examine the muscle specific ageing and development of muscle mechanical properties
2. To compare the effects of ageing between muscles with predominantly different fiber type compositions, anatomical function and location (EDL versus Diaphragm).

Hypotheses

1. The rate of muscle development and the reduction in performance due to ageing will be muscle specific.
2. Diaphragm muscle will reach physiological maturity faster than EDL due to its importance in respiratory function and thus potentially highlighting a hierarchy of muscle development.
3. The age related reduction in maximal muscle power output (and corresponding muscle activation and relaxation time) will be greater than the reduction in maximal force.
4. The effect of ageing on fatigue resistance will be muscle specific; generally muscle will show a decrease in the ability to maintain force

Study 5 (Chapter 8)-**Does the Ergogenic Benefit of Caffeine Change with Age? The Effect of Physiological Concentrations of Caffeine on the Power Output of Maximally Stimulated Mouse EDL and Diaphragm Muscle**

Aims

1. To examine the direct effect of physiologically relevant concentrations of caffeine on isolated skeletal muscle from development and with increasing age.

2. To assess if any potential change in the ergogenic benefit of caffeine is muscle specific (EDL versus Diaphragm).
3. To uniquely examine whether the direct effect of physiological concentrations of caffeine can significantly enhance diaphragm muscle power output and thus potentially improve respiratory performance.

Hypotheses

1. The direct treatment of muscle with 70μM caffeine will significantly enhance muscle power output in developing and ageing muscle.
2. The level of caffeine response will be significantly reduced in developing muscle, will peak at physiological maturity, and again decrease with increasing age.
3. The sensitivity of skeletal muscle to70μM caffeine treatment will be muscle specific and during ageing these differences will relate to differences in the rate of ageing between EDL and diaphragm muscles.

3. General Methods

Animals

The ethics committee of Coventry University approved the use of animals in the present series of studies. In all cases female white mice (strain CD1 mice, Charles River, UK) were used and were bred and kept in house at Coventry University. Unless stated otherwise, animals were housed in groups of 8-10, were kept on the same diet (STF maintenance diet and water *ad libitum*) throughout the duration of this body of work, and were subjected to 12 hour light-dark cycles. A qualified vet checked all animals regularly and any animals deemed unhealthy were removed.

Mice selected for experimental use were weighed and then killed by cervical dislocation in accordance with British Home Office Animals (Scientific Procedures) Act 1986, Schedule 1.The hind limbs of each mouse were skinned and the limb with the target muscle removed and put into refrigerated (4°C) oxygenated (95% O_2; 5% CO_2) Krebs-Henseleit solution of composition (mM) NaCl 118; KCl 4.75; $MgSO_4$ 1.18; $NaHCO_3$ 24.8; KH_2PO_4 1.18; glucose 10; $CaCl_2$ 2.54; pH 7.55 at room temperature (James *et al.* 2005).

Skeletal Muscle Dissection and Preparation

The limb with the target muscle or the rib cage with the full diaphragm muscle intact, was then pinned out at approximately its resting length in fresh and frequently changed oxygenated Krebs-Henseleit solution at room temperature (19-24^{o}C). Generally, for each muscle preparation the tendon and a small piece of bone were left attached at the proximal and distal ends. As shown in Figure 3.1 aluminium foil T-clips were wrapped around each tendon leaving the bone at the back of the clip to help minimise tendon slippage when the muscle is producing force (James *et al.* 2005). The aluminium foil T-clips were used to attach the muscle preparation inside the muscle bath (Fig 3.2.).

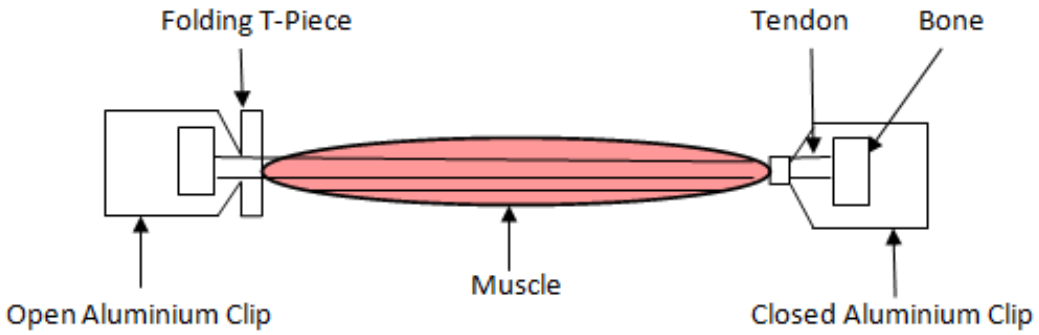


Figure 3.1. Preparing the muscle isolation - The muscle was isolated with the tendon and a small piece of bone intact at the proximal and distal ends. Aluminium clips were attached around the tendons by folding the T-Pieces to prevent tendon slippage and to subsequently secure the preparation within the muscle bath.

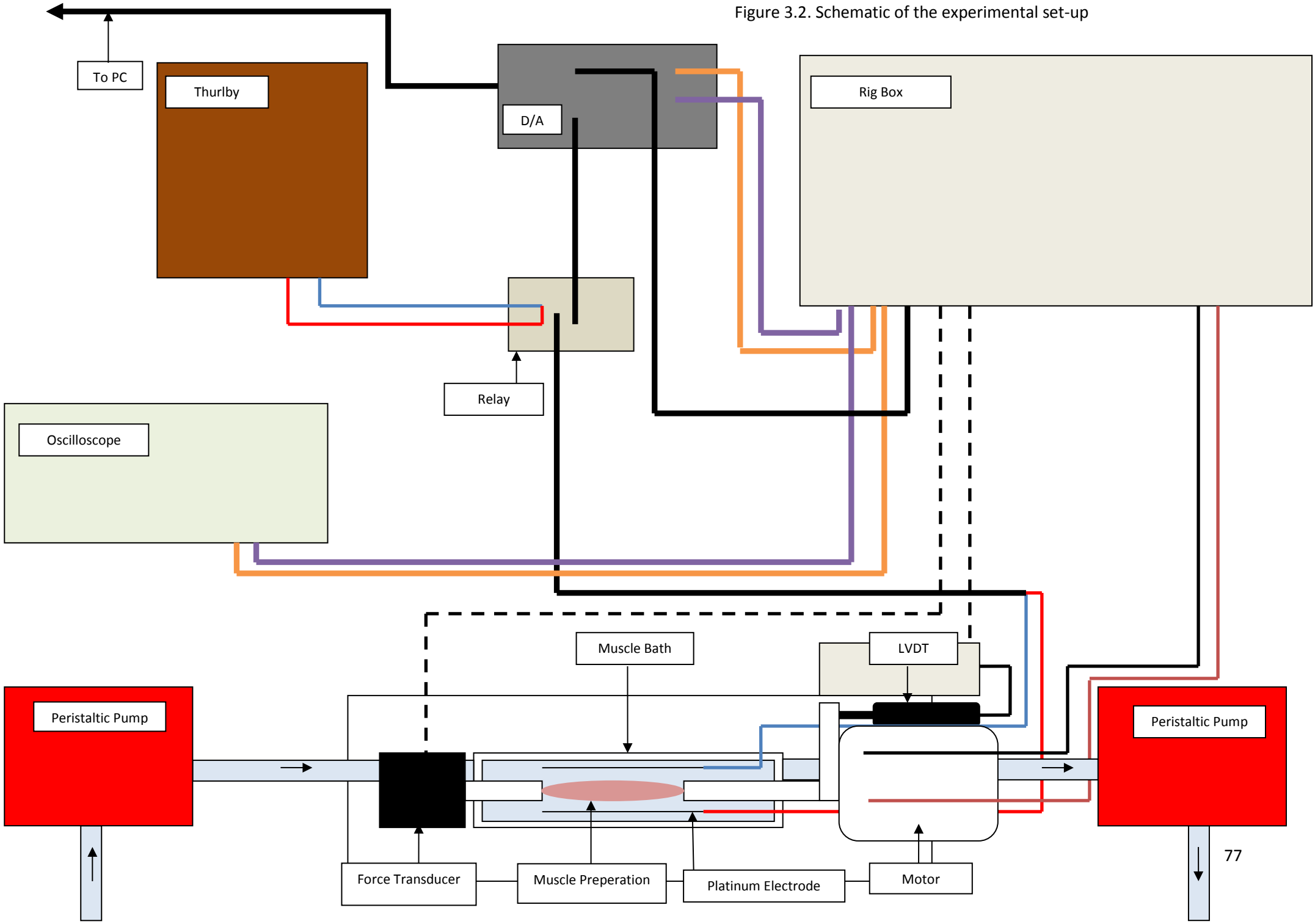
Experimental Set-Up (Figure 3.2.)

The experimental set-up is a custom designed muscle rig that allows alterations in muscle stimulation parameters and length, permitting not only the measurement of isometric force, but dynamic power output (i.e. work loops). Once dissected the muscle preparation was attached using the foil T-clips to a 25g force transducer (UF1, Pioden Controls Ltd, UK) and a motor (V201, Ling Dynamic Systems, UK) via crocodile clips in the muscle bath. Position of the motor arm was detected via a Linear Variable Displacement Transformer (DFG5.0, Solartron Metrology, UK). Changing the distance between the force transducer and the motor altered muscle length inside the muscle bath. Peristaltic pumps maintained a constant flow of oxygenated Krebs-Henseleit solution into the muscle bath at a physiological temperature (36-37^{o}C). Krebs was pumped from a reservoir of Krebs where the target temperature was maintained via a heater/cooler bath (Grant LTD6G, Grant Instruments Ltd, UK). The temperature inside the bath was continuously measured with a digital thermometer (Checktemp C, Harvard Apparatus, UK). The force transducer measured the force the muscle produced during electrical stimulation and the signal from the

force transducer was fed, via the rig box, to the oscilloscope to provide a visual representation, and to the PC running the custom written Testpoint software (Testpoint, CEC, Massachusetts, USA), via the data acquisition board(KPCI3108, Keithley Instruments, Ohio, USA). The muscle preparation was electrically stimulated via the parallel platinum electrodes in contact with the circulating fluid; the stimulation amplitude was controlled by the Thurlby. The rig box processes and amplifies the force and length signals, the oscilloscope is used for a rapid check of both force and length signals and during isometric studies it is used to measure maximal twitch and tetanus force and tetanus times. A PC running the Testpoint software controlled the variables of stimulation and length. These parameters were muscle specific and greater detail of this and the specific experimental protocol are discussed at length in each chapter.

All muscles were subjected to a series of isometric studies followed by work loop experiments.

Figure 3.2. Schematic of the experimental set-up



Calibration of the Force Transducer

The force transducer was regularly calibrated by hanging known weights from it. Once these weights were applied, the change in electrical signal was measured on the oscilloscope and the data plotted as in Figure 3.3. Determining the slope and intercept of this data allowed the calculation of the calibration (in mN/V) of the force transducer.

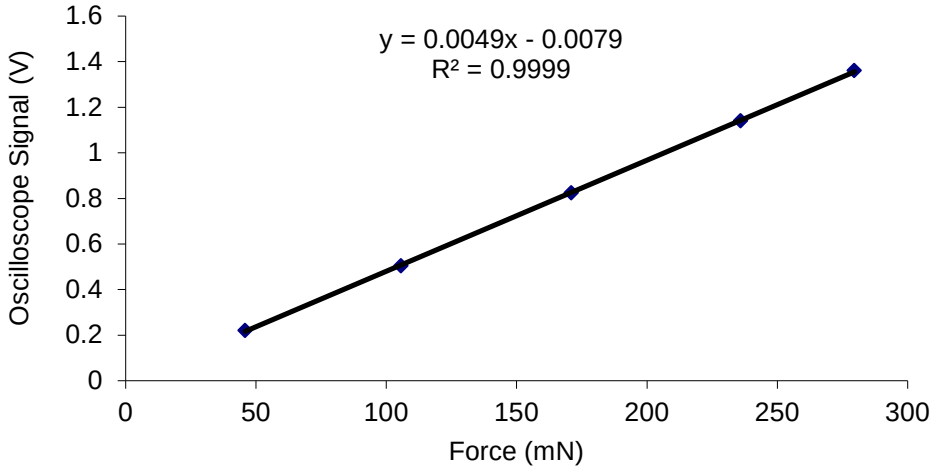


Figure 3.3. - Calibration of the force transducer; y = force production (dependant variable); x = voltage recorded on the oscilloscope (independent variable)

Mechanical Measurements of *in vitro* Skeletal Muscle Performance

10 minutes prior to the commencement of testing the muscle was allowed to equilibrate to the new environment. The primary mechanical measures of isolated muscle performance in the present research consisted of isometric and work loop assessments. Initially each muscle was subjected to a single stimulus to evoke a twitch response; muscle length and stimulation amplitude(generally 12-16V for soleus; 14-18V for EDL; 10-16V for diaphragm) were varied until maximal twitch force was produced. The duration of electrical stimulation (generally 320 ms for soleus; 200 ms for EDL, 250ms for diaphragm) and the stimulation frequency (generally 140Hz for soleus and diaphragm; 200Hz for EDL) were then increased to evoke a maximal tetanic force response. A 5 minute rest period was

imposed between each tetanus in order to ensure the muscle had sufficient recovery time. Figure 3.4.demonstrates the effect of increasing stimulation frequency on isometric tetanus response. Using this isometric force trace, measurements of activation time (time to half peak tetanus; THPT) and relaxation time (time from last stimulus to half tetanus relaxation; LSHR) were made. The stimulation amplitude, duration of electrical stimuli and stimulation frequency that elicited maximal tetanic response was muscle specific and is defined in each experimental chapter.

The muscle length that corresponded to maximal isometric twitch and tetanus force was measured using an eyepiece graticule fitted to a microscope and was defined as L_0.Mean muscle fiber length was calculated as 85% and 75% of L_0 for soleus and EDL muscle respectively (James *et al.* 1995), this is generally used as an estimation of fiber length. As no such estimate of fiber length exists for diaphragm the physical measurement taken was used as L_0.The optimal length for force generation under isometric contraction may not directly correspond to the optimal length for maximal net work generated by the work loop technique; however this measurement has been used as an estimation in previous work loop studies (Josephson, 1985: Askew *et al.* 1997; James *et al.* 1995; 1996; 2004; 2005; Vassilakos *et al.* 2009).

The work loop technique assesses the ability of the muscle to produce power whilst undergoing cyclical length changes (Josephson; 1985; James *et al.* 1996; 2005). Here the muscle was held at L_0 and the stimulation amplitude and frequency parameters that yielded maximal tetanic force were employed. Via movement of the motor arm each muscle was subjected to four sinusoidal length change cycles per set at a total symmetrical strain of 0.10, thus the muscle lengthened by 5% from L_0 followed by a shortening to 5% shorter than L_0 before returning back to L_0.Length change cycle frequency, electrical stimulation phase and burst duration were optimised to produce maximal work loop power. Typically the cycle frequency and strain employed were a gained from estimates of the values that elicit maximal power and/or are attainable *in vivo* as suggested in previous research (James *et al.* 1995; Askew *et al.* 1997; Vassilakos *et al.* 2009).

Generally a burst duration of 100ms was found to elicit maximal power output in EDL, consistent with the findings of James *et al.* (2004; 2005). The burst duration dictates the number of stimuli that the muscle receives during the work loop; optimising this duration maximises power output. Commanlya burst duration of 65 ms was found to elicit maximal power output in soleus, consistent with the findings of James *et al.* (2004; 2005) and Vassilakos *et al.* (2009). However, on occasions when subjecting soleus to a submaximal stimulation frequency (i.e. 40Hz), the burst duration was lengthened to 76 ms adding a further stimulus during the shortening phase of the work loop. This adjustment was determined by examining power output values. If the muscle is was too active during lengthening there is greater resistance to elongate the muscle back to resting length and therefore a decreased net power output. The strain of 0.10 was fixed for all experiments and was based on previous estimations of the strain required for production of maximal power in the muscle preparations used in the present thesis (Altringham and Young, 1991; James *et al.* 1995; Vassilakos et al. 2009). The stimulation phase shift dictates that stimulation of the muscle starts 10 ms prior to the muscle reaching maximal length, therefore with a stimulus duration of 65ms in soleus, stimulation continues until 45 ms prior to the muscle reaching its shortest length.

In order to measure muscle power output, the muscle was subjected to four work loop sets. Commonly the second loop of each set of four work loops was used as an indicative measure for each trial as it produced the highest work. Following optimisation of the length and stimulation parameters, these variables were kept constant and a 10-minute rest between each trial was enforced in order to allow maximal recovery time (James *et al.* 2004). The specifics of each experimental protocol to examine the ergogenic effect of caffeine on muscle power output are explained in depth in each experimental chapter.

Data were sampled at a rate of 10 kHz and then a work loop was formed, by plotting force against length, the area of which represents the net work done by the muscle during a single length change cycle (Josephson, 1985).Typical work loop shapes in response to altered stimulation frequency are

shown in Figure 3.4. The use of the isometric and work loop techniques, as well as the specific protocols for each experiment are explained in much greater depth in the individual studies within the thesis.

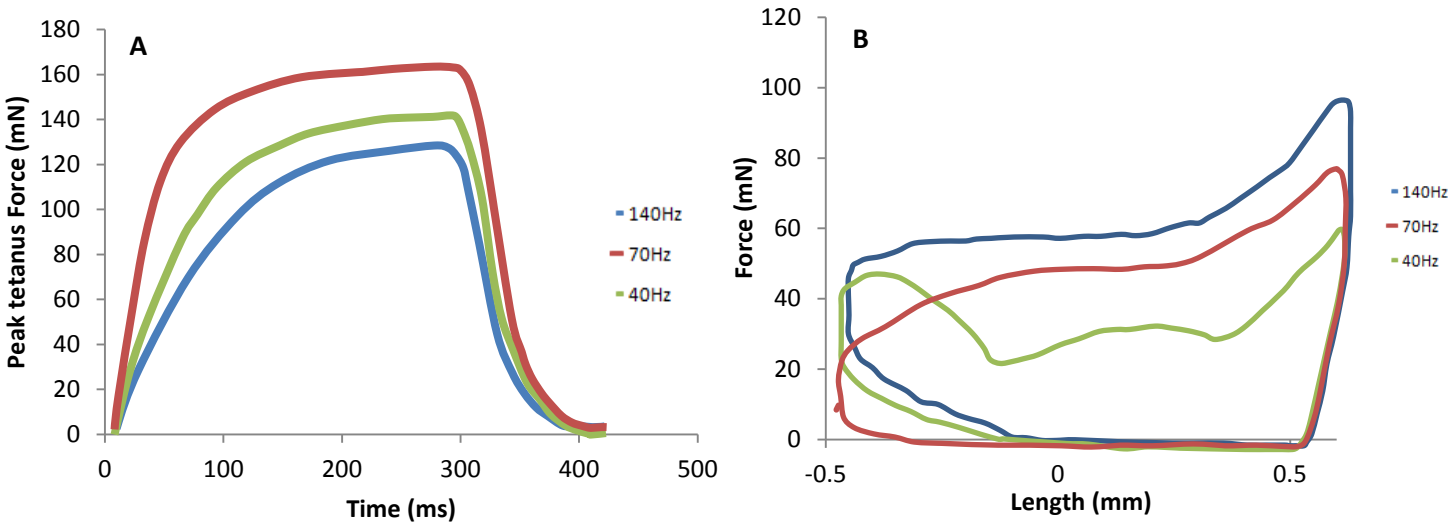


Figure 3.4. – Typical tetanus response following 320ms stimulation duration (A) and work loop shape at 5Hz cycle frequency (B) for mouse soleus muscle stimulated at 140Hz, 70Hz, and 40Hz stimulation frequency

Muscle Mass Measurements and Dimension Calculations

At the end of the experiment the tendons were removed leaving the muscle intact. Following this the muscle was blotted on tissue paper to remove excess fluid. The muscle was then placed on an electronic balance (Mettler Toledo B204-S, Zurich, Switzerland) to determine the wet muscle mass to the nearest 0.0001g. Mean muscle cross-sectional area was calculated from mean fiber length, muscle mass and an assumed muscle density of 1060 kg m^{-3} (Mendez & Keys, 1960). Isometric stress was calculated as force divided by mean muscle cross-sectional area. Muscle power output was normalised to muscle mass to express power as $W.kg^{-1}$.

The Correction Factor

Before the commencement of the experiments of the present thesis, initial investigation of the response of the isolated muscle preparations over time were made in order to assess if muscle power output was altered in a predictable manner. The results indicated (Figure 3.5.) a linear reduction in mean maximal soleus muscle power output over time that is largely attributed to a build-up of an anoxic core causing the slow death of the contractile proteins (Barclay, 2005). The predictable liner relationship demonstrated here forms the bases of the correction factor used in caffeine treated muscles.

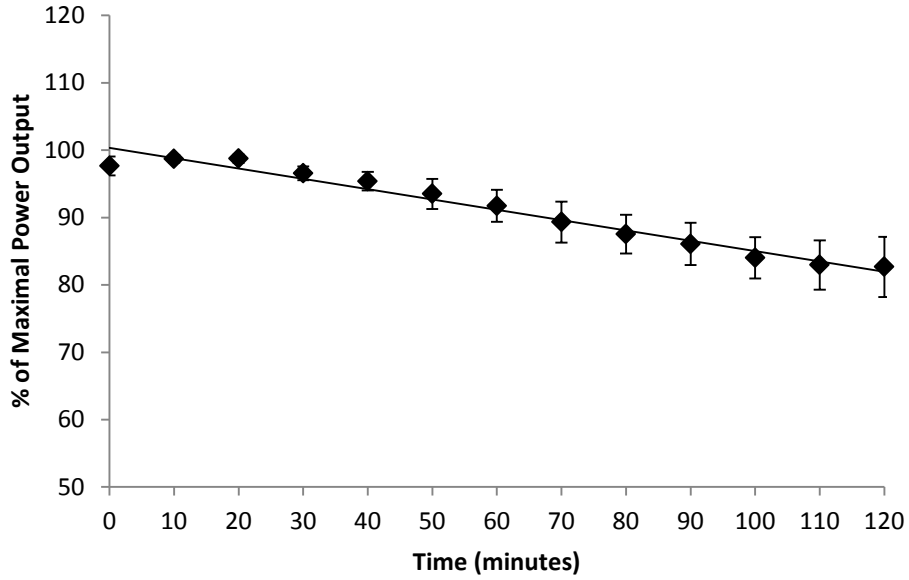


Figure 3.5. – Decrease in mean power output over time in non-treated mouse soleus muscle [Data represented as mean ± SE] n=6. A first order regression line was fitted to the data (y= -0.153x + 100.3; r^2 = 0.996).

In order to avoid deterioration in muscle performance masking the effects of caffeine, a 1st order regression equation was calculated using the control data and washout data to identify the linear relationship between muscle power output and time. This regression equation was then used to

determine theoretical control muscle power output for each time point during caffeine treatment (see example in Figure 4.3.3.).

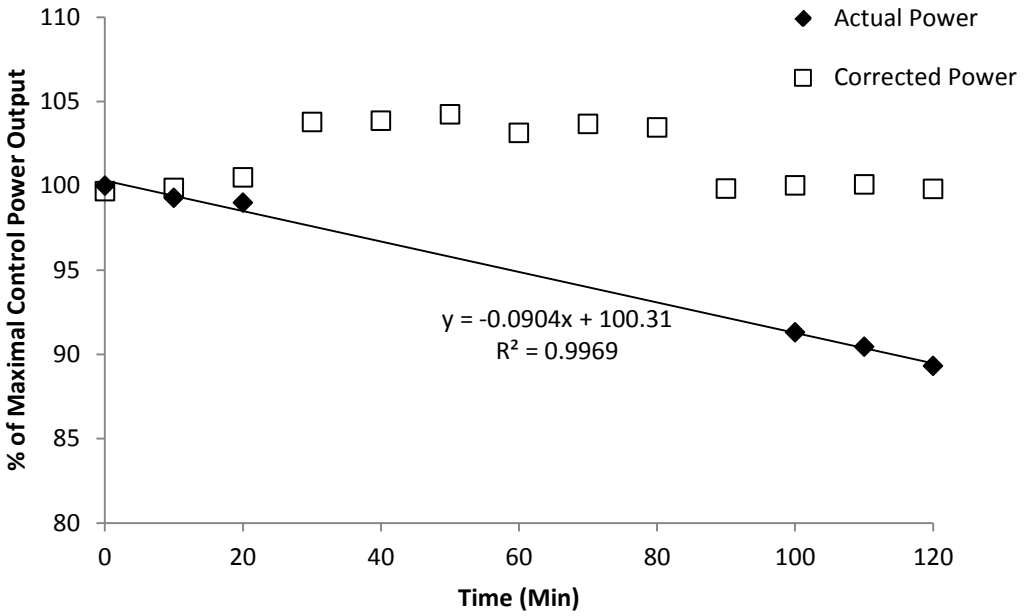


Figure 3.6. – A typical example of the decline in actual muscle power output where the regression equations is used to calculate corrected power output

4. The Effects of Physiological Concentrations of Caffeine on the Power Output of Maximally and Sub Maximally Stimulated Mouse EDL (Fast) and Soleus (Slow) Muscle

Caffeine Improves Maximal & Submaximal Muscle Performance

Modified from Publication in the Journal of Applied Physiology:

Tallis, J., James, R.S., Cox, V.M., Duncan, M.J. (2012) 'The effect of physiological concentrations of caffeine on the power output of maximally and sub maximally stimulated mouse EDL (fast) and soleus (slow) muscle' *Journal of Applied Physiology* 112 64-71

**This chapter is referred to as Tallis et al. (2012) throughout the thesis as per publication.*

4.1 Abstract

The ergogenic effects of caffeine in human exercise have been shown to improve endurance and anaerobic exercise performance. Previous work has demonstrated that 70μM caffeine (physiological maximum) can directly increase mouse extensor digitorum longus (EDL) muscle power output (PO) in sprint like activity by 3%. Our study used the work loop technique on isolated mouse muscles to investigate whether the direct effect of 70μM caffeine on PO differed between: 1) maximally and different sub maximally activated muscle; 2) relatively fast (EDL) and relatively slow (soleus) muscles; 3) caffeine concentrations. 70μM caffeine treatment resulted in significant improvements in PO in maximally and sub maximally activated EDL and Soleus ($P < 0.03$ in all cases). For EDL the effects of caffeine were greatest when the lowest, submaximal, stimulation frequency was used ($p<0.001$). 140, 70 and 50μM caffeine treatments resulted in significant improvements in acute PO for both maximally activated EDL (3%) and soleus (6%) ($P < 0.023$ in all cases), however there was no significant difference in effect between these concentrations ($P>0.420$ in all cases). Therefore, the ergogenic effects of caffeine on power output were higher in muscles with a slower fiber type ($P < 0.001$). Treatment with 35μM caffeine failed to elicit any improvement in PO in either muscle ($P > 0.72$ in both cases). Caffeine concentrations below the physiological maximum can directly potentiate skeletal muscle power output. This caffeine induced increase in force could provide similar benefit across a range of exercise intensities with greater gains likely in activities powered by slower muscle fiber type.

Key Words: Force, Work Loop, Skeletal Muscle, Sprint, Activation Level

4.2. Introduction

Caffeine (common name for 1,3,7-trimethylxanthine) is a powerful ergogenic aid that has been extensively studied for its effects in improving exercise capacity (Graham, 2001). *In vivo* and *in vitro* studies have found enhancements in endurance exercise performance, power, and fatigue recovery, accredited primarily to the effects of caffeine on the CNS (Kalmar & Cafarelli, 2004). Despite its documented popularity as a performance enhancer, aiding training and competition, caffeine presently still falls short of the World Anti-Doping Agency's prohibited list (Word Anti-Doping Agency 2011). Reviews by Graham (2001) and Davis and Green (2009) suggest that caffeine can enhance performance during endurance (activity lasting greater than 30 minutes), power and strength activities.

Following ingestion, caffeine can be readily absorbed into the blood stream with peak plasma concentration occurring 30-60 minutes after ingestion (Lorist & Topps, 2003). Its hydrophobic nature allows free passage of caffeine across all biological membranes resulting in distribution throughout all the tissues of the body (Magkos & Kavouras, 2005). Caffeine is also able to diffuse from the cerebral circulation across the blood brain barrier, entering the cerebrospinal fluid in sufficient quantity to promote pharmacological effects (McCalll *et al.* 1982; Wilkinson & Pollard, 1993; Fredholm *et al.* 1999; George, 2000). Caffeine is considered to act centrally as a competitive adenosine receptor antagonist, increasing transmission via dopamine D_2 receptors (Fredholm *et al.* 1999; Snyder *et al.* 1981). Lorist and Tops (2003) explored behavioural and performance responses to caffeine ingestion demonstrating an increase in response to stimuli, an elevated state of arousal and a decreased rate of perceived exertion. Caffeine has also been demonstrated to have a direct effect on skeletal muscle by acting as an adenosine receptor antagonist on A1 receptors directly on the skeletal muscle membrane and/or by binding to the RYR receptors of the SR resulting in altered excitation contraction coupling (Bhat *et al.* 1997; Damiani *et al.* 1996; Fredholm *et al.* 1999; Rossi *et al.* 2001).

Early *in vitro* studies demonstrated the direct potentiating effects of caffeine on acute muscle twitch and tetanus force, however many of these studies used supraphysiological, millimolar, concentrations of caffeine which would be toxic to humans (Luttgau & Oetliker, 1967; Endo *et al.* 1970; Huddart, 1968; Weber & Herz, 1986; Fredholm *et al.* 1999). The primary mechanism by which caffeine can promote enhanced force output in skeletal muscle is believed to be via interference of excitation contraction coupling (Davis & Green, 2009). It has been established that the specific mechanism of action is alteration of intramuscular ion handling, primarily via an increased concentration of Ca^{2+} within the intracellular space (Magkos & Kavouras, 2005). However, little is known about whether variation in physiological conditions, such as intensity of exercise and caffeine dosage, will alter the direct response of muscle to caffeine during human physical activities. Tarnopolsky & Cupido (2000) reported that 6 $mg.kg^{-1}$ body mass (approximately 60µM in blood plasma) of caffeine enhanced involuntary evoked skeletal muscle force in human subjects at low, but not high stimulation frequencies. This was attributed to a potentiation of calcium release at lower stimulation frequencies promoting a greater influx of Ca^{2+} in the presence of caffeine. However, no previous *in vitro* study has directly tested isolated muscle to determine whether the enhancement of force and power production in skeletal muscle, due to caffeine treatment, is greater at lower stimulation frequencies. Such findings would be of practical benefit to athletes, as they would indicate the types of physical activities in which the ergogenic effects of caffeine were greatest. In the context of the present study it is considered that a lower stimulation frequencies, SR calcium concentration will be greater. In light of the conclusions of Tarnopolski and Cupido (2000) it is likely that caffeine will have a more pronounced effect on sub maximally activated muscle due to a greater potentiation of calcium release.

James *et al.* (2004; 2005) were the first to test the effect of physiologically relevant concentrations of caffeine (70µM human *in vivo* maximum, Graham, 2001) using the work loop technique. They found a small but significant, 2-3%, increase in mean net power output in maximally activated isolated mouse EDL (fast muscle), attributed to increased force production during shortening. 70µM

caffeine treatments had no significant effect on delaying the onset of fatigue or enhancing fatigue recovery. Evidence from use of millimolar concentrations of caffeine (which would represent toxic blood plasma concentrations in man, Fredholm, 1999), has shown that potentiation occurs to a greater extent in relatively slower muscle e.g. soleus (Fryer & Neering, 1989; Rossi *et al.* 2001; Wondmikun *et al.* 2006). This has largely been accredited to differences in Ca^{2+} kinetic properties (Magkos & Kavouras, 2005). However, no previous study has tested whether there is a difference between muscle fiber types in the direct effect of physiological concentrations of caffeine on power output. A dose dependant effect on direct muscle performance has further been demonstrated with high 0.07-20 millimolar concentrations of caffeine (Fryer & Neering, 1989; James *et al.* 2005), however this response has not been investigated over physiologically relevant caffeine concentrations, therefore, there are currently no studies to indicate the dosage of caffeine required for humans to maximise power output in muscle during physical activity.

The present study aims to investigate whether maximal physiological concentrations (70µM) of caffeine directly affect the power output of isolated skeletal muscle during brief bouts of cyclical activity, being the first such study to compare between: 1) maximally and sub maximally activated muscle; 2) relatively fast extensor digitorum longus (EDL) and relatively slow soleus muscles; 3) a range of concentrations (35-140µM) of caffeine. In light of the previous research it is hypothesised that muscle power output of both EDL and soleus muscle will be increased in response to 70µM caffeine treatment, and there will be no dose dependant effect when used over a physiological range. Furthermore the performance enhancing effect of caffeine will be greater in soleus muscle compared to EDL. Finally it is considered that stimulating muscle at a submaximal intensity in the presence of 70µM caffeine will promote further power potentiation than at maximal intensity.

4.3. Materials and Methods

A more detailed account of the methods is given in the general methods section (chapter 3).

Soleus and EDL muscle were isolated from the right hind limb of 8-10 week old mice (body mass = 30.2 ± 0.81g, mean ± SE, *n* = 108). Once placed in to the muscle rig, isometric studies were conducted. Muscle length and stimulation amplitude were optimised (12-16V for soleus; 14-18V for EDL) to produce maximal isometric twitch force. Following this, at a pre-determined burst duration (320 ms for soleus; 200 ms for EDL); stimulation frequency was optimised to induce maximal isometric twitch force (normally 140Hz for soleus; 200Hz for EDL). Tetanic responses were also measured at two submaximal stimulation frequencies (70 & 40Hz for soleus; 150 & 100Hz for EDL). The lowest stimulation frequency reduced muscle power output below 60% in both muscles. A 5-minute rest period was imposed between each tetanus in order to ensure that the muscle had sufficient recovery time.

The work loop technique was used to determine maximal and submaximal (stimulation frequencies 140, 70 & 40Hz for soleus; 200, 150 & 100Hz for EDL) work loop power output at 5Hz cycle frequency for both soleus and EDL. 5Hz cycle frequency has previously been shown to elicit maximal power output in mouse soleus muscle and is attainable in running mice (Askew *et al.* 1997; James *et al.* 1995). 5Hz was also used for EDL preparations in order to enable a direct comparison with soleus muscles, however maximal power output for EDL is achieved at 10Hz cycle frequency (James *et al.* 1995).

In order to examine an acute effect of caffeine the muscle preparations were subjected to four work loops at 10-minute intervals over a 130-minute duration (Fig 4.3.1.). A 10-minute rest interval was enforced between each set of four work loops, here, and throughout the remainder of the protocol, in order to allow ample time for recovery (James *et al.* 2004). The protocol consisted of three control measurements in standard Krebs-Henseleit solution, followed by six measurements in Krebs-

Henseleit solution containing 70µM caffeine, concluding with a washout period of four measurements in standard Krebs-Henseleit solution (Fig 4.3.1.). In order to test for a possible interaction between caffeine and simulation frequency this procedure was repeated using 140, 70 or 40Hz and 200, 150 or 100Hz stimulation frequencies for soleus and EDL respectively (n = 8 in all cases). To examine the effects of altered caffeine concentration the same procedure was followed, however the concentration of caffeine added to the Krebs-Henseleit solution was altered to 140, 50 or 35µM (Fig 4.3.1.). i.e. caffeine concentration was reduced until no effect was demonstrated; we also wanted to determine if doubling the suggested physiological maximum would result in a greater response..

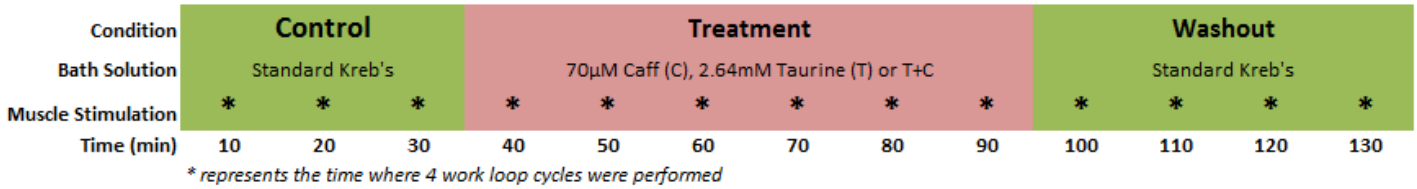


Figure 4.3.1. - Schematic of the work loop protocol to examine the ergogenic effect of caffeine on the acute muscle power output of maximally and sub maximally stimulated mouse EDL and soleus muscle

At the end of the experiment the muscle was detached from the rig, tendons removed, then weighed in order to calculate isometric stress ($kN.m^{-2}$) and normalised muscle power ($W.kg^{-1}$).

Statistical Analysis of the Data

Single factor analysis of variance (ANOVA) were performed in SPSS (Version 16, SPSS inc., IL, USA) in order to investigate the difference in isometric stress and work loop power between EDL and soleus muscles. Further single factor ANOVA's were performed in order to examine the effect of stimulation frequency, before any caffeine treatment, on: isometric stress in soleus; isometric stress in EDL; work loop power in soleus; work loop power in EDL.

Prior to commencement of testing, muscle stress and power output at 140Hz, 70Hz and 40Hz for soleus and 200Hz, 150Hz and 100Hz for EDL were measured in all the preparations used. Two factor ANOVA's (2x3 ANOVA's) were conducted on this data to test for significant differences between stimulation frequencies and the caffeine treatment categories in which the preparations were subsequently placed. Therefore, stimulation frequency and caffeine treatment category were used as the fixed factors and power output as the dependant variable. Tukey post hoc tests were performed for stimulation frequency where any significant differences were found.

Prior to testing the effect of 70µM caffeine over different stimulation frequencies, there was no significant difference in stress and power output between caffeine treatment categories in soleus and EDL (ANOVA p <0.65 in all cases) prior to caffeine treatment. Prior to testing the effects of different caffeine concentrations there was no significant difference in stress and power output between treatment categories in EDL (ANOVA p = 0.723). In soleus the 50µM treatment group produced significantly more stress than 35µM group (ANOVA Tukey p<0.001), however there was no significant difference between any of the other treatment groups (Tukey p>0.505 in all cases). For soleus and EDL there was no significant difference in power output between the treatment categories (p=0.695 in both cases). A reduction in stimulation frequency resulted in a reduction in stress and power in all treatment groups for both EDL and soleus (ANOVA p<0.001 in all cases). Therefore it is fair to conclude the preparations were of similar quality prior to treatment.

As explained in the general methods section (Chapter 3) a regression equation used to determine theoretical control muscle power output for each time point during caffeine treatment. The range of regression coefficients were R^2 = 0.002-0.9972, the level of significance between these regressions varied between p < 0.001 - 0.883. Typically muscle preparations that demonstrated a degree of deterioration over time showed significant regression coefficients normally exceeding 0.8 (p<0.05 in each case). Preparations that were stable over time had low regression coefficients and the effect of the correction was minimal.

A single factor ANOVA was conducted on each treatment group to determine any difference between prior treatment control and post treatment washout. For soleus and EDL muscles there was no significant difference between the prior and post treatment controls. Therefore, it is assumed that after the caffeine treatment the muscles returned to their previous state and any changes in performance during treatment were solely the effects of caffeine. These control data were pooled and subsequent analysis was conducted comparing caffeine treatment directly against controls.

The effects of stimulation frequency (100Hz, 150Hz, 200Hz for EDL; 40Hz, 70Hz, 140Hz soleus) and caffeine treatment (70uM, control) on soleus power output were tested in 2-factor (3x2) ANOVA. The same statistical test was conducted in a separate 2-factor (3x2) ANOVA for the EDL muscle. In order to test for a significant effect of caffeine concentration a further 2 factor (2x3) ANOVA was conducted separately for soleus and EDL. Again power output was the dependent variable with caffeine treatment (70μM caffeine or control) and caffeine concentration as the fixed factors. A significant interaction between concentration and treatment was identified in EDL treated with 70μM caffeine at different stimulation frequencies and both soleus and EDL treated with altered caffeine concentrations (two factor (2x3) ANOVA $p<0.015$ in all cases), therefore we conducted a single factor ANOVA on each treatment group to determine the effect of caffeine compared to control.

Results were interpreted as significant when $p< 0.05$. Values are displayed as mean ± standard error.

4.4. Results

EDL produced significantly greater maximum isometric stress and greater maximal WL PO than soleus (Table 4.4.1; single factor ANOVA main effect $p<0.001$ in both cases). Reducing stimulation frequency resulted in a significant reduction in isometric tetanic stress (to 87.8% and 66.4% of maximal for soleus when stimulated at 70 and 40Hz and to 91.1% and 69.6% of maximum for EDL when stimulated at 150Hz and 100Hz) for both soleus and EDL (Table 4.1.1; two factor (2x3) ANOVA main effect $p<0.001$ in both cases). A reduction in stimulation frequency also resulted in a significant decrease in maximum work loop stress (to 64.3% and 42.9% of maximum for soleus when stimulated at 70 and 40Hz and to 87.1% and 59.5% of maximum for EDL when stimulated at 150Hz and 100Hz) in soleus and EDL (Table 4.1.1; two factor (2x3) ANOVA main effect $p<0.001$ in both cases).

Table 4.4.1. – The mean effect of altered stimulation frequency on tetanus stress and work loop power in mouse EDL and soleus.

	Soleus		
Twitch Stress ($kN.m^{-2}$)		32.7±2.6	
	*	*	*
Stimulation frequency	40Hz	70Hz	140Hz
Tetanus Stress ($kN.m^{-2}$)	125.8±11	166.2±11.5	189.4±11.9
Max Work Loop PO (W/kg)	13.6±1.2	20.4±1.9	31.7±1.8
	EDL		
Twitch Stress ($kN.m^{-2}$)		66.2±6.2	
	*	*	*
Stimulation frequency	100Hz	150Hz	200Hz
Tetanus Stress ($kN.m^{-2}$)	209±22.43	273.9±24.3	300.5±23.2
Max Work Loop PO (W/kg)	50.7±5	74.2±6.4	85.2±7.1

*[Data represented as Mean ± SE; * indicate significant differences]*

Effects of Stimulation Frequency and 70µM Caffeine Treatment on Muscle Power Output

70µM caffeine treatment of soleus elicited significantly greater power output than controls in all cases (Figure 4.4.1; single factor ANOVA main effect p=0.02). Caffeine elicited a mean peak power output increase of 6.4%, 6.2% and 5.7% for 140Hz, 70Hz and 40Hz stimulation frequencies respectively. There was no significant difference in the effect of caffeine between stimulation frequencies (Figure 4.4.1; two factor (2x3) ANOVA main effect p=0.093).

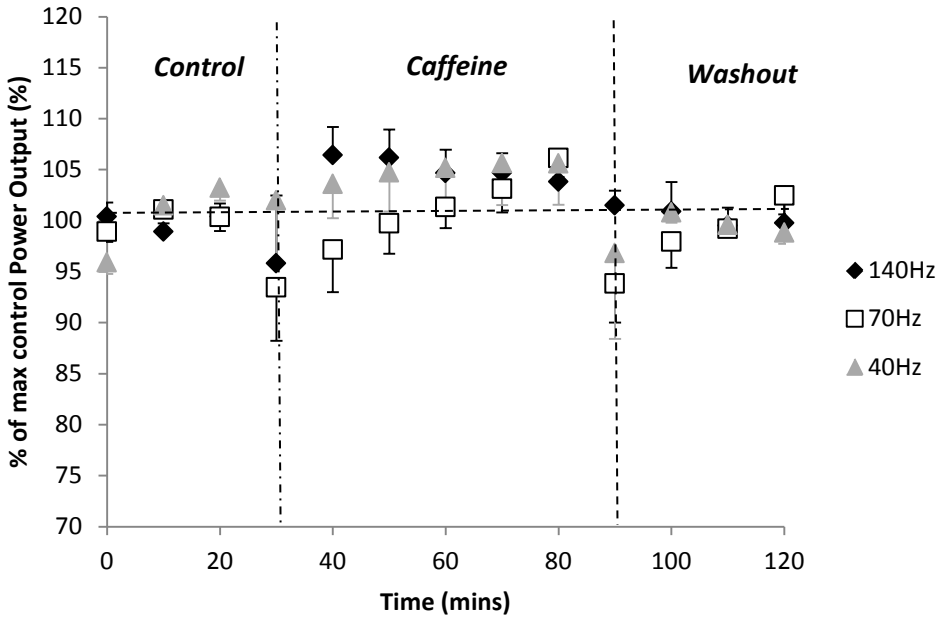


Figure 4.4.1. – The mean acute effect of 70µM caffeine on work loop power output in mouse soleus muscle at 140Hz, 70Hz, and 40Hz stimulation frequencies [Data represented as mean & SE] n=8 in each case.

70µM caffeine treatment of EDL elicited significantly greater power output than controls (Figure 4.4.2; single factor ANOVA main effect p<0.005 in all cases). Caffeine elicited a mean increase in peak power output of 3.3%, 4.2%, and 6.7% for 200Hz, 150Hz and 100Hz stimulation frequencies respectively. There was no significant difference in the effect of 70µM caffeine between 200Hz and 150Hz (Figure 4.4.2; Tukey p=0.976). However, 70µM caffeine treatment elicited a significantly greater increase in PO at 100Hz compared to 200Hz and 150Hz (Figure 4.4.2; two factor (2x3) ANOVA Tukey p<0.005 in both cases). The decline in net power output in the transition between control and caffeine treatments and again at the

transition between caffeine treatments in washout, is caused by altered environmental conditions due to altered solution. At these time points there is a period of a few seconds where the muscle is not fully saturated with solution and time is required for the temperature to equilibrate.

A set of responders and a set of non-responders (those showing no noticeable improvement in response to treatment) were evident in soleus (Figure 4.4.3) and EDL (Figure 4.4.4) in all the treatment groups besides 100Hz sub maximally stimulated EDL. Caffeine treated EDL, stimulated at 100Hz, showed no obvious non-responders to the treatment (Figure 4.4.4C).

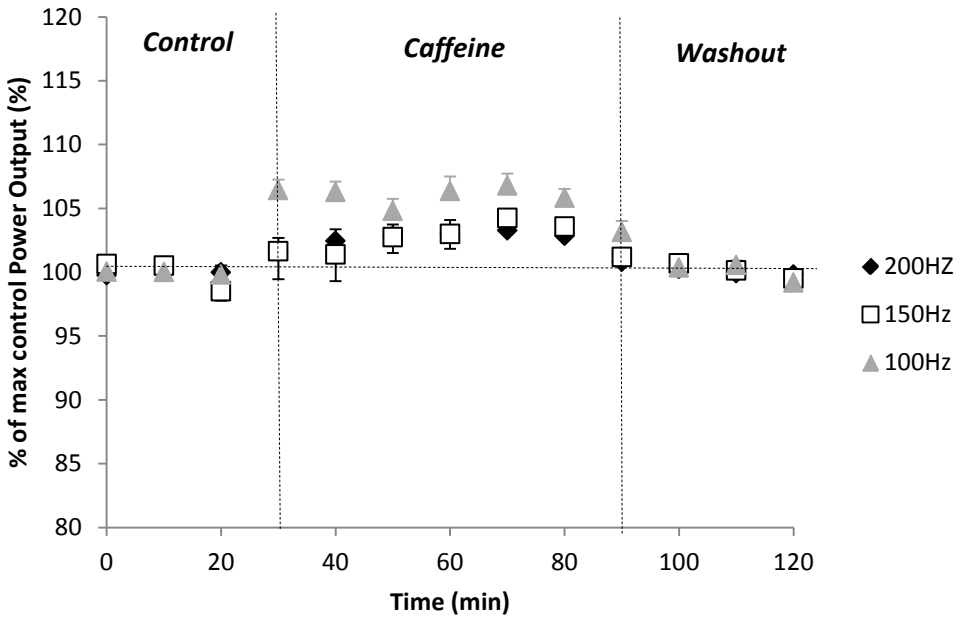


Figure 4.4.2. – The mean acute effect of 70µM caffeine on work loop power output in mouse EDL muscle at 200Hz, 150Hz, and 100Hz stimulation frequencies [Data represented as mean & SE] n=8 in each case

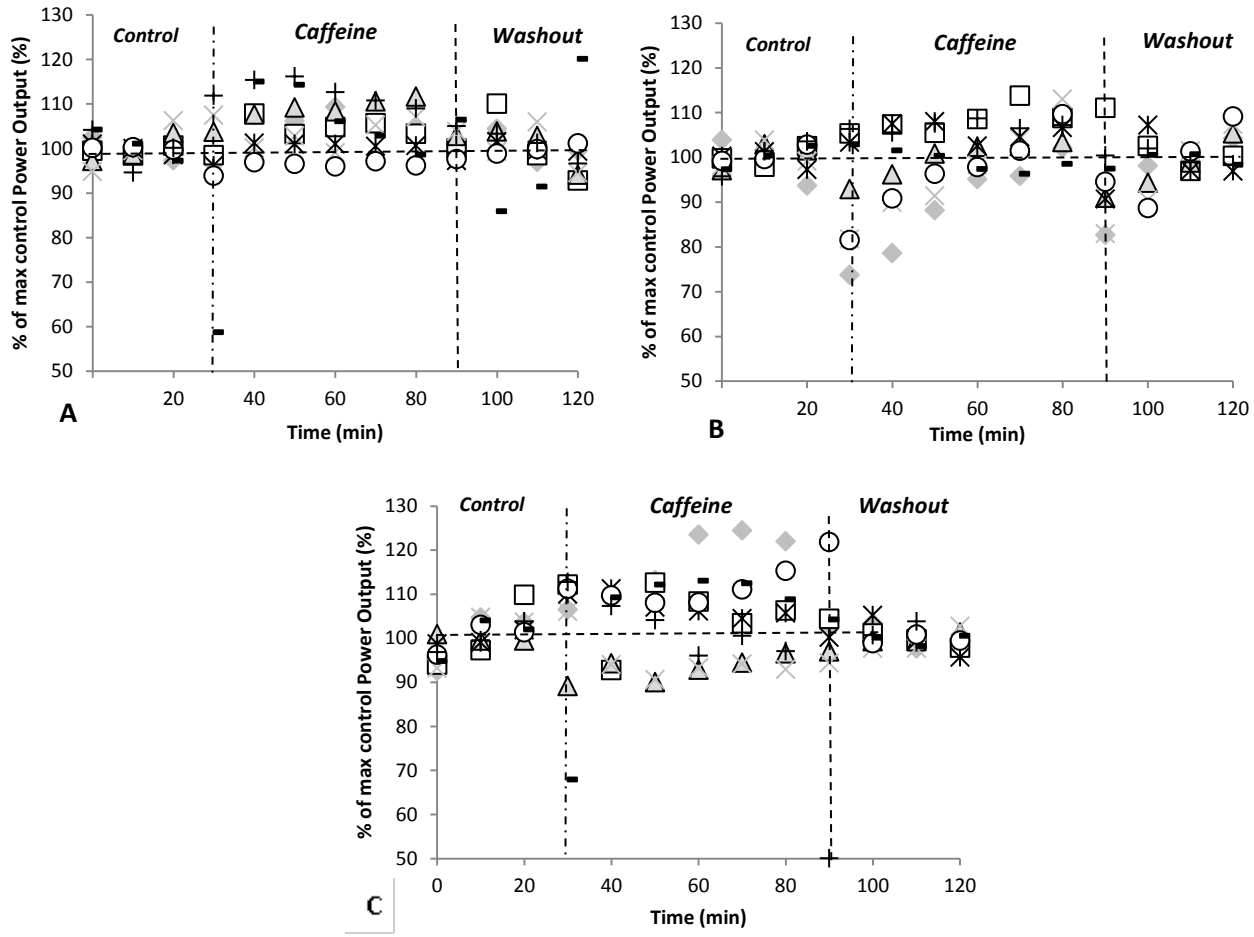


Figure 4.4.3. – The acute effect of 70μM caffeine on work loop power output identifying the differences in response to caffeine between individual mouse soleus muscles at 140Hz(A), 70Hz(B), and 40Hz(C) stimulation frequencies. The magnitude of response to caffeine varies between individuals, with some individuals (non-responders) showing no change in power output [Symbols represent different individuals].

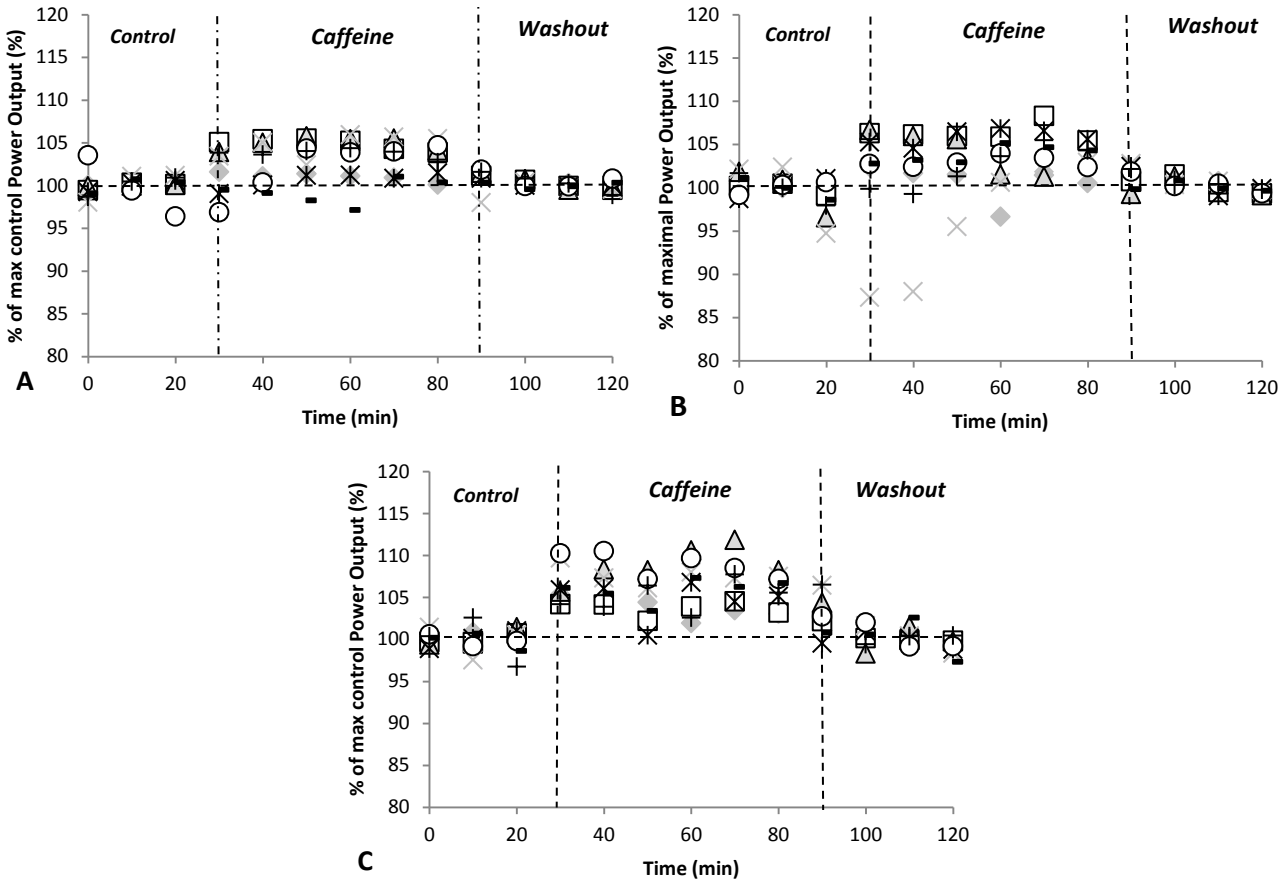


Figure 4.4.4. – The acute effect of 70µM caffeine on work loop power output identifying the differences in response to caffeine between individual mouse EDL muscles at 200Hz(A), 150Hz(B), and 100Hz(C) stimulation frequencies. The magnitude of response to caffeine varies between individuals, with some individuals (non-responders) showing no change in power output [Symbols represent different individuals].

The Effect of 35, 50, 70 & 140µM Caffeine Treatment on WL Power Output

Treatment of soleus muscle with 140µM, 70µM and 50µM caffeine resulted in a significant increase in maximal power of up to 6% (Fig 4.4.5 single factor ANOVA main effect $p<0.015$ in all cases). Treatment using 35µM caffeine failed to significantly increase soleus muscle's maximal PO (Fig 4.4.5 single factor ANOVA main effect $p= 0.072$). There was no significant difference in the increase in PO between 140µM, 70µM and 50µM caffeine treatments (Fig 4.4.5; two factor (2x3) ANOVA Tukey $p< 0.473$ in all cases).

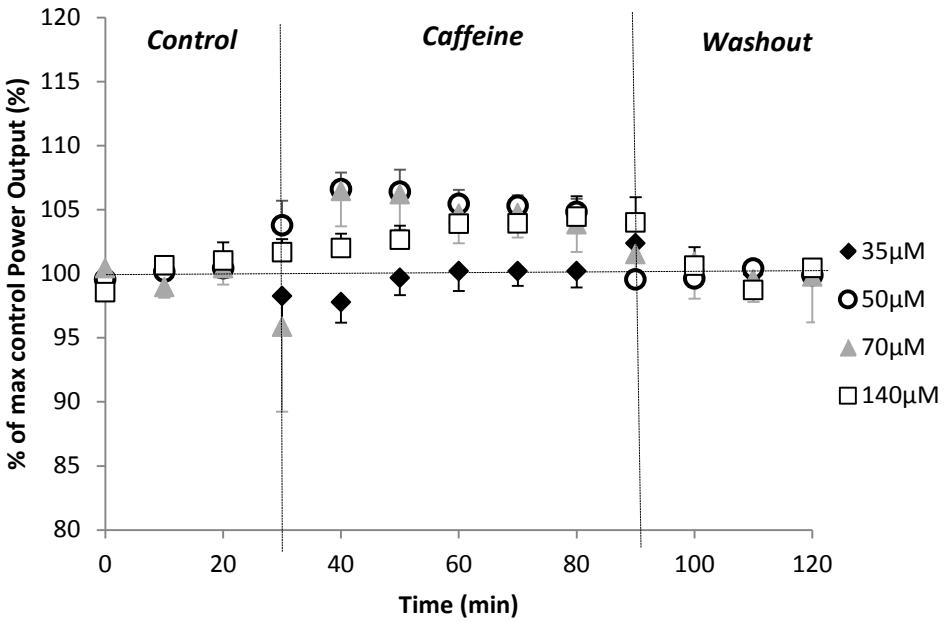


Figure 4.4.5. - The mean acute effect of 140, 70, 50 & 35µM caffeine treatment on work loop power output of mouse soleus muscle maximally stimulated at 140Hz stimulation frequency [Data represented as mean & SE] n=10 for 35, 50 and 140µM n=8 for 70µM.

Treatment of EDL muscle with 140µM, 70µM and 50µM caffeine resulted in a significant increase in mean maximal power of up to 3.3% (Figure 4.4.6; single factor ANOVA main effect $p<0.022$ in all cases). Treatment using 35µM caffeine failed to significantly increase EDL muscles maximal PO (Figure 4.4.6; single factor ANOVA main effect $p= 0.341$). There was no significant difference in the increase in PO

between 140µM, 70µM and 50µM caffeine treatments (Figure 4.4.6; two factor (2x3) ANOVA Tukey *p*> 0.421 in all cases).

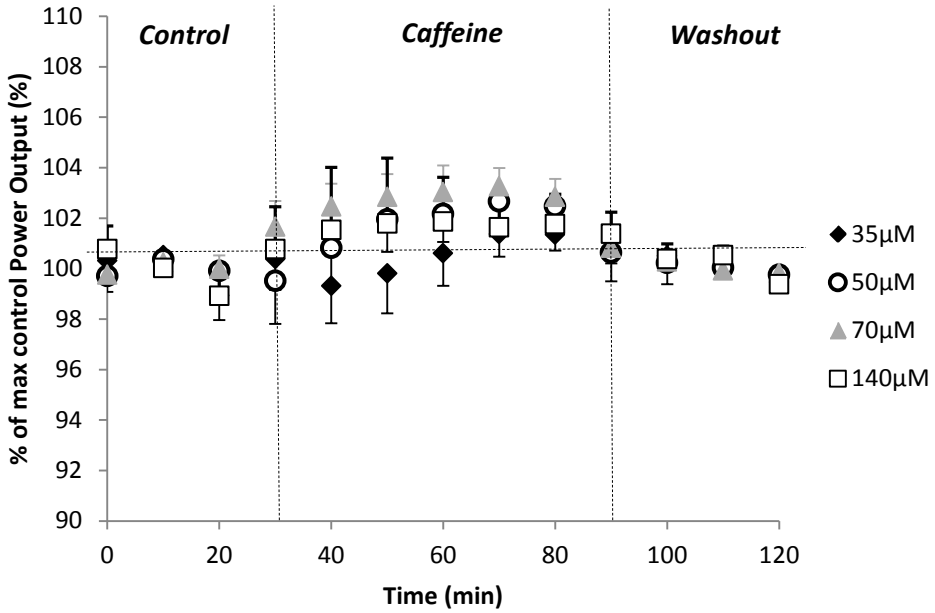


Figure 4.4.6. - The mean acute effect of 140, 70, 50 & 35µM caffeine treatment on work loop power output of mouse EDL muscle maximally stimulated at 140Hz stimulation frequency. [Data represented as mean & SE] n=10 for 35, 50, 140µM; n=8 for 70µM.

As there was no significant difference in response between 140µM, 70µM and 50µM caffeine treatments these results were pooled and soleus was compared against EDL. Treatment of soleus muscles with 50µM - 140µM caffeine resulted in a significantly greater increase in power output (4.7%) compared to EDL (2.5%) muscle (Figure 4.4.7; ANOVA two factor (2x3) main effect p<0.001)

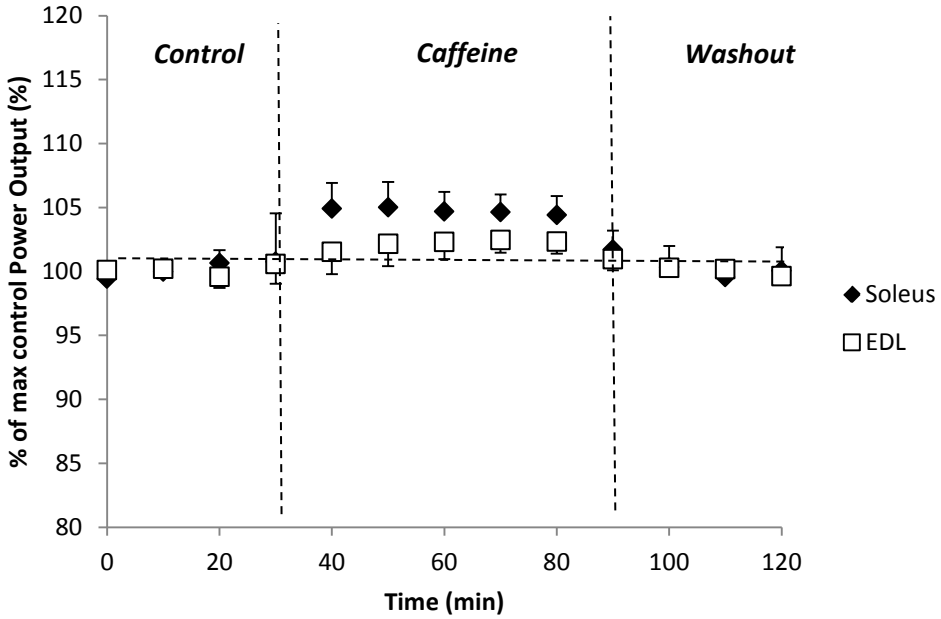


Figure 4.4.7. - The mean effect of caffeine treatment on acute maximal power output of mouse EDL and soleus muscle [140,70& 50µM data pooled for each muscle; Data represented as mean & SE; n = 28 in each case]

4.5. Discussion

The mean maximal isometric tetanic stress was 189 ± 12 kN $m^{-2}$and 300 ± 23 kN m^{-2} for soleus and extensor digitorum longus (EDL) respectively (Table 4.4.1). This is similar to soleus but notably higher for EDL stresses previously reported by James *et al.* (1995; 2004) and Vassilakos *et al.* (2009) in studies using similar methods. The mean control maximal power output was 31.7 ± 1.8 W kg^{-1} and 85.2 ± 7.1 W kg^{-1} (Table 1) again similar to the values reported by James *et al.* (1995; 2004), Askew *et al.* (1997) and Vassilakos *et al.* (2009). Any differences in stress and power output between studies could be attributed to muscle fiber type differences due to variation in strain and age of the mice and the environmental conditions at which they were kept. Variation in muscle mass and length will also affect the maximal stress and power that the muscle can achieve.

The effects of 70μM caffeine on muscle power output at maximal and sub maximal stimulation frequencies.

Treatment of mouse EDL and soleus muscle with 70μM caffeine elicited significantly greater net WL power output. A mean increase in soleus power output of approximately 6% occurred at each stimulation frequency (Fig 4.4.1). In EDL the caffeine induced enhancement of net power output decreased with increased stimulation frequency from 6.7% at 100Hz to 3.3% at 200Hz (Fig 4.4.2). The ergogenic benefit was not significantly different between stimulation frequencies in soleus, however a lower stimulation frequency (100Hz) produced significantly greater force in EDL compared to higher stimulation frequencies. Therefore, in EDL the effects of caffeine on power output were greater when the lowest, submaximal, stimulation frequency was used.

A caffeine treatment induced elevation in muscle power output supports the finding of James *et al.* (2005) who also used a physiologically relevant 70μM caffeine concentration to treat maximally stimulated EDL. The 3.3% increase obtained in EDL in the present study using the same parameters is similar to the 2-3% increase reported by James *et al.* (2005), but markedly lower than the 6.4% power improvement seen in

soleus in the present study. These results from the present study suggest that in mammals physiological levels of caffeine treatment will directly induce small increases in power output in short term high intensity activity (e.g.100m sprint in athletics) however, it seems likely that caffeine will have greater ergogenic benefit during lower intensity sporting activities that are primarily powered by slow muscle fiber types.

It has long been established that caffeine can alter excitation-contraction coupling (Magkos & Kavouras, 2005). The mechanism by which this increase in power output has occurred in the present study can be attributed to the ability of caffeine to alter intramuscular ion handling. The mechanism for this action of caffeine is still unclear, however, it is believed that caffeine operates directly as an adenosine receptor antagonist on A1 receptors on the skeletal muscle membrane and/or binds to RYR receptors of the SR as shown in *vitro* with 10mM caffeine treatment and in RYR -/- mice (Damiani *et al.* 1996; Bhat *et al.* 1997; Fredholm *et al.* 1999; Rossi *et al.* 2001). These processes probably result in a combination of improved opening of the RyR2 channels of the SR stimulating a greater release of Ca^{2+} into the intracellular space, an increase in myofibrillar Ca^{2+} sensitivity, a decrease in the sensitivity of the SR Ca^{2+} pump, and an increased SR Ca^{2+} permeability. Consequently the rate of Ca^{2+} efflux from the intracellular space back to the SR may be significantly slower resulting in a greater basal and activated intracellular Ca^{2+} concentration, hence increased relaxation time (Allen *et al.* 1989; Allen & Westerblad, 1995). The work loop shapes for both EDL and soleus (Fig 4.4.8) show that caffeine treatment caused a direct increase in muscle force during shortening, however, no appreciable change in relaxation time can be seen. As the muscle was only subjected to 4 work loop cycles it is unlikely that the proposed increase in basal Ca^{2+} between stimulations will occur over this short time period. Fryer and Neering (1989) reported that the primary effect of caffeine (0.2 – 20 mM) on Ca^{2+} transient was an increase in basal and stimulus evoked release of Ca^{2+} accompanied by an elevation of the plateau phase leading to an increase in twitch and tetanus force in rat EDL and soleus. Magkos & Kavouras (2005) further suggested that if Ca^{2+} is released from SR at a quicker rate then this will result in quicker initiation of the Ca^{2+} induced Ca^{2+} response mechanism. The primary consequence of these effects is enhanced cross-bridge kinetics

initially allowing faster and greater availability of the actin binding sites due to a quicker movement of troponin-C, thus promoting greater formation of cross bridges and hence higher force production. Maintaining an elevated concentration of intracellular Ca^{2+} between stimulus intervals will result in a higher net quantity of calcium when the muscle receives further stimuli. Muscle force is dependent on the concentration of free Ca^{2+}, thus a caffeine-induced elevation in this manner will result in greater force production. With an increased intracellular Ca^{2+} concentration and a decreased sensitivity of the SR Ca^{2+} pump the time required to regain intracellular resting concentration of Ca^{2+} and replenish the SR stores of Ca^{2+} will be significantly elongated (Allen & Westerblad, 1995).

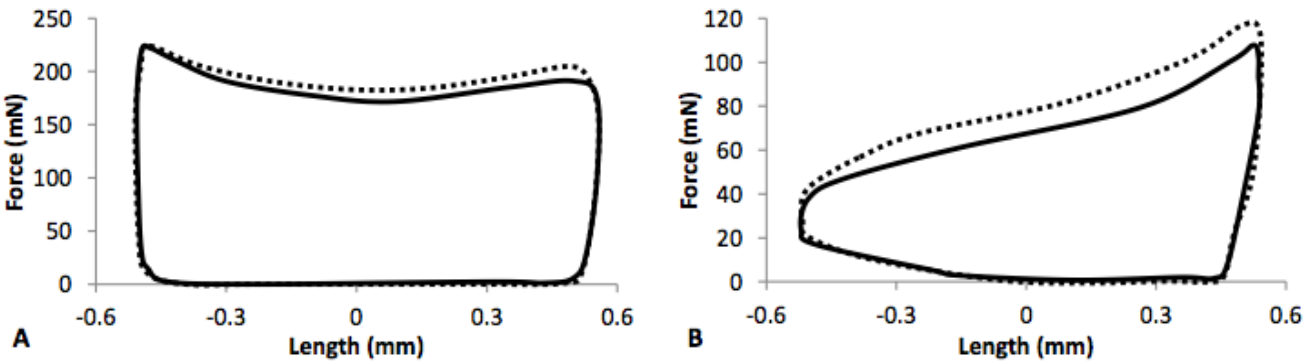


Figure 4.4.8. – Typical effects of caffeine treatment on work loop shapes in mouse EDL (A) and soleus (B) stimulated maximally at 5Hz cycle frequency [solid line = control work loop; dashed line = caffeine treated work loop]

Generally *in vivo* and *in vitro* studies report the benefit of caffeine as a group mean (Allen & Westerblad, 1995; James *et al.* 2004; Norager *et al.* 2005; Bridge& Jones, 2006), however a degree of inter-individual variability in response is common and studies have shown that not all individuals show a performance improvement (Bridge & Jones, 2006; Davis & Green, 2009; James *et al.* 2005). In the present study there were also individual muscles that showed no appreciable change in power output in response to caffeine (Fig 4.4.3 & 4.4.4). To the authors knowledge caffeine has not been demonstrated to cause a reduction in acute muscle force, therefore from a human perspective, micromolar concentrations of caffeine in human blood plasma can most likely have direct beneficial or negligible effects on skeletal muscle performance.

Tarnpolsky & Cupido (2000) suggested that at a sub maximal stimulation frequencies caffeine would promote greater release of Ca^{2+}. The present study doesn't fully support this finding, as there were no significant increases in soleus muscle power output with decreased stimulation frequency. EDL showed a similar response when 200Hz (maximal) was compared against 150Hz, however, a significant enhancement in muscle power did occur at 100Hz. For this treatment group there appeared to be no 'non responders' to the caffeine treatment (Fig 4.4.4; C) hence the mean increase in power output was significantly higher than at 200Hz and 150Hz. As the precise mechanism of the action of caffeine is still unknown we are unable to suggest that a greater number of responders occur at lower stimulation frequencies. Overall our findings suggest that there is a limit to the level of calcium influx that caffeine promotes and further highlights the need for greater investigation into the mechanisms of the response.

The effects of 35, 50, 70 and 140µM concentrations of caffeine on maximal force production

140µM, 70µM and 50µM caffeine treatment resulted in significant improvements in mean power output of mouse soleus (up to 6%; Fig 4.4.5) and EDL muscle (up to 3.3%; Fig 4.4.6). There were no significant differences in the level of ergogenic benefit between each concentration. Treatment of soleus and EDL with 35µM caffeine failed to potentiate force.

Human physiological concentrations of caffeine are very rarely above 70µM with common plasma levels being between 20-50µM (Fredholm *et al.* 1999; Graham, 2001). The present study indicates that there appears to be a threshold level of caffeine concentration, below which there is no response and above which there is no further effect of increasing concentration within the physiological range. The effect of 6 or 9 $mg.kg^{-1}$ body mass caffeine treatment on 2000m rowing performance was considered by Bruce *et al.* (2000). The Low dose caffeine trial resulted in a significant 1.3% improvement in time to complete the 2000m whilst the high dosage resulted in a significant 1% improvement. Above the physiological range Fryer and Neering (1989) demonstrated a dose dependant potentiation of twitch force in EDL (1- 5 mmol l^{-1}) and soleus (0.2 – 1 mmol l^{-1}) fibers of rat. James *et al.* (2005) reported that fatigued mouse soleus and EDL produced significantly greater peak stress and power output during shortening of cyclical

contractions with 10mM caffeine treatment compared to those treated with 70µM caffeine and controls in conjunction with the present findings these previous studies suggest there is no dose dependant effect of caffeine over the human physiological range.

The effects of caffeine on different muscle fiber types

The ergogenic benefit was significantly greater in mouse soleus (4.7%) compared to EDL (2.5%; Fig 4.4.7). This is comparable to previous evidence, using non-physiological concentrations of caffeine, where fast twitch fibers yielded a greater response to caffeine treatment than slow twitch fibers. Rossi *et al.* (2001) reported a greater response to 2-30mM caffeine concentrations in mouse soleus compared to EDL. Fryer and Neering (1989) further demonstrated that soleus was more sensitive to a lower dose (200µM) of caffeine compared to EDL. This can be attributed to muscle specific differences in Ca^{2+} kinetic properties and muscle specific expression of RYR isoforms between type I and II fiber types (Magkos & Kavouras, 2005). Rossi *et al.* (2001) reported that mouse skeletal muscle RYR3 receptors have a greater sensitivity to caffeine than RYR1. It has been established that muscles with the greatest response have a greater quantity of RyR3. A higher quantity of RyR3 is evident in soleus muscle explaining the elevated response of soleus in the present study (Rossi *et al.* 2001).

When relating these results in a broader context to human performance it should be considered that caffeine has a shorter half-life in rodents and differences in metabolism also occur between rodents and primates (Fredholm *et al.* 1999). Fibers treated with caffeine may *in vivo* be modulated to produce the same power as controls with the activation of fewer muscle fibers. *In vivo* the pattern of fiber stimulation along with length change waveforms are likely to be manipulated throughout movement in order to maximise muscle economy and prevent the onset of fatigue (Wakeling 2005). However, these differences are unlikely to affect the overall findings of the study.

In conclusion physiological levels of caffeine (50 & 70µM) can directly enhance mouse soleus and EDL muscle power output during short-term cyclical activity. Further to this caffeine appears to have no dose

dependant effect on skeletal muscle when used over a relatively small concentration range (50-140µM). The current study shows that caffeine doses lower than the physiological maximum can produce significant improvements in muscle force. Treatment with 35µM caffeine showed no appreciable change in the power output of either soleus or EDL, therefore it is assumed that a relatively high concentration of caffeine is needed to evoke physiological benefit directly at the skeletal muscle. From the results of the current study it appears that the extent of caffeine-induced potentiation of power output is unlikely to differ between muscles stimulated sub maximally compared to maximally.

5. Does a Physiological Concentration of Caffeine (70µM) Affect Endurance in Maximally or Sub Maximally Stimulated Mouse Soleus (Slow) muscle?

Modifeid from publication in The Journal of Physiological Sciences

Tallis, J., James, R.S., Cox, V.M., and Duncan, M.J. (2013) 'The effect of a physiological concentration of caffeine on the endurance of maximally and submaximally stimulated mouse soleus muscle.' *The Journal of Physiological Sciences* 63 (2) 125-132.

**This chapter is referred to as Tallis et al. (2013) throughout the thesis as per publication.*

5.1. Abstract

The use of caffeine as an ergogenic aid to promote endurance has been widely studied, with human literature showing the greatest benefit during sub maximal muscle activities. Recent evidence from isolated muscle studies suggests that the acute treatment of skeletal muscle with physiological concentrations of caffeine (70μM maximum) will directly potentiate force production. The aims of the present study are: firstly to assess the effects of a physiological concentration (70μM) of caffeine on endurance in maximally activated mouse soleus (relatively slow) muscle; secondly to examine whether endurance changes when muscle is activated sub maximally during caffeine treatment. Maximally stimulated soleus muscle treated with 70μM caffeine resulted in a significant (17.6%) decrease in endurance. In contrast, at a sub maximal stimulation frequency caffeine treatment significantly prolonged endurance (by 19.2%).Findings are activation dependent such that during high frequency stimulation caffeine accelerates fatigue, whereas during low frequency stimulation caffeine delays fatigue.

Key Words: Activation Level, Ergogenic Aid, Endurance, Power, Isolated Muscle, Skeletal Muscle, Work Loop

5.2. Introduction

The ergogenic effect of caffeine in man has been studied extensively and performance-enhancing effects have been clearly established in endurance, strength, and power based activities (Graham 2001, Davis & Green 2009). Recent work by James *et al.* (2004; 2005) and Tallis *et al.* (2012: Chapter 4) have demonstrated a direct effect of physiologically relevant (70μM) caffeine concentrations on isolated mammalian muscle. However, further investigation is needed in order to assess the direct effects of 70μM caffeine on skeletal muscle endurance.

Skeletal muscle fatigue is defined as a reduction in force generating capacity associated with muscle activity, and is further associated with a reduced relaxation rate and changes in the force-velocity relationship (Fitts 1994; Askew *et al.* 1997). The aetiology of muscle fatigue is influenced by the intensity and duration of physical activity and mechanistically arises from a combination of central and peripheral physiological actions (Allen *et al.* 2008; Favero 1999). The primary mechanism for the reduction in skeletal muscle force relates to a reduction in the efficiency of excitation-contraction coupling arising from a fatigue induced impairment in sarcoplasmic reticulum (SR) Ca^{2+} release and a reduction in myofibrillar sensitivity to Ca^{2+} (Fabiato & Fabiato 1978; Godt & Nosek 1988; Millar & Homsher 1990; Lamb *et al.* 1992; Allen *et al.* 1995; Allen *et al.* 2008).

It has been proposed that caffeine may be effective at reducing skeletal muscle fatigue during human endurance based activities via a reduction of the effects brought on by fatigue (Graham 2001, Magkos & Kavouras 2005). Caffeine acts as an adenosine receptor antagonistic, specifically at the A1 receptors on the skeletal muscle membrane, but can also bind directly to ryanodine receptors (RYR) of the SR (Daminani & Margreth 1994; Fredholm *et al.* 1999; Rossi 2001). The treatment of isolated skeletal muscle with high, millimolar, concentrations of caffeine has been demonstrated to affect Ca^{2+} handling, primarily by inducing an increased Ca^{2+} concentration in the intracellular space (Allen *et al.* 1989; Allen & Westerblad, 1995; Westerblad & Allen 1991; Allen *et al.* 2007).

Many *in vitro* studies have demonstrated the direct force potentiating effect of caffeine using millimolar concentrations that would be toxic for human consumption (Luttgau & Oetliker 1967; Huddart 1968; Weber & Herz 1968; Endo *et al.* 1970; Damiani & Margreth 1994). Previous work by Tallis (chapter 4), James and co-workers (2004, 2005) was the first to show a direct force potentiating effect of caffeine using physiologically relevant concentrations (70μM maximum). However, the direct significance of such concentrations of caffeine on muscle endurance requires greater exploration (James *et al.* 2004; 2005; Tallis *et al.* 2012; (chapter 4) Damiani & Margreth 1994).

The effects of 70μM caffeine on the time to fatigue on net power output of maximally stimulated mouse EDL (relatively fast) muscle has previously been determined by James *et al.* (2005), where treatment failed to elicit any significant effect. Tallis *et al.* (2012; chapter 4) was the first to demonstrate that 70μM caffeine treatment caused greater acute improvements in muscle force and power in soleus (relatively slow) when compared to EDL (6% and 3% improvements, respectively), however, it is not known whether micromolar caffeine can affect endurance in soleus muscle. The greater acute response of soleus muscle to caffeine suggests that caffeine treatment is more likely to offset the fatigue induced reduction in sarcoplasmic reticulum Ca^{2+} release in soleus than in EDL, which may result in a prolonged endurance. Therefore, the present study aims to build on the previous work by James *et al.* (2005) by assessing the effects of 70μM caffeine on the endurance of mouse soleus muscle. As the aetiology of fatigue is not only influenced by muscle fiber type but the intensity of activity, and slow oxidative fibers are of primary importance during prolonged submaximal exercise, this study will uniquely examine if the effect of caffeine is altered in soleus muscle fatigued at high compared with low frequencies of stimulation. Furthermore findings from Tarnopolsky & Cupido (2000) in a whole body human study, suggest that the ergogenic effect of caffeine may be greater at lower stimulation frequencies due to a greater potentiation of SR calcium release. Although no effect was observed in our previous work examining one off muscle power output (Tallis *et al*. 2012: Chapter 4), exposing the muscle to caffeine over prolonged contracture may evoke differences in action between maximally and sub maximally fatigued muscle.

James *et al.* (2005) further reported a reduced recovery from fatigue following the treatment of EDL with micromolar caffeine. Therefore, the effect of recovery from fatigue will be analysed in the present study in order to determine whether caffeine treatment reduces the subsequent recovery of mouse soleus muscle from fatigue. Caffeine treatment may potentially affect the post fatigue recovery of muscle power output due to a potential increase in muscle stiffness resulting in muscle damage (James *et al.* 2004). Furthermore, if caffeine treatment results in improved muscle performance the muscle will be working at a relatively greater intensity compared to controls and hence recovery may be prolonged.

In contrast to the findings of James *et al.* (2005) (no effect of 70μM caffeine on maximally fatigued mouse EDL) the present study predicts that the treatment of mouse soleus muscle with 70mM caffeine will significantly enhance fatigue resistance. Furthermore it is suggested that the caffeine enhanced improvement in endurance will be significantly greater in sub maximally stimulated muscle.

5.3. Materials and Methods

A more detailed account of the methods is given in the general methods section (chapter 3).

Soleus muscle was isolated from the right hind limb of 8-10 week old mice (body mass = 30 ± 1.6g, *n* = 32). Once dissected the muscle was placed in the muscle bath and circulated with oxygenated Krebs-Henseleit solution at 36±0.4^{o}C. Muscle length and stimulation amplitude (12-16V) were optimised to elicit maximal isometric twitch force. Maximal isometric tetanic force was measured by subjecting the preparation to a burst of electrical stimuli (320 ms) at a pulse width of 1.5 ms. Stimulation frequency was optimised to yield maximal tetanic force (usually 140Hz), by measuring the response over a stimulation frequency range (100Hz-160Hz). Following this the tetanic force response was measured at two sub maximal stimulation frequencies (40 and 70Hz). A 5-minute rest period was imposed between each tetanus in order to ensure that the muscle had sufficient recovery time. Isometric tests were carried out in the same way on all muscle preparations used in this investigation. Thereafter, each muscle preparation was subjected to work loop experiments.

Prior to the fatigue protocol, muscle power output was measured using the work loop technique at stimulation frequencies that yielded maximal (140Hz; high) and sub maximal (40Hz; low) power in all the preparations used at the previously defined parameters. As in chapter 1, during work loop experiments soleus muscle power was measured at 5Hz cycle frequency using a strain of 0.10, and a fixed phase shift of -10ms. Each muscle was subjected to sets of four work loops at 10-minute intervals; the second loop of each set of four was used as an indicative measure of performance. A 10-minute rest interval was enforced between each set of four work loops, here, and throughout the remainder of the protocol, in order to allow ample time for recovery (James *et al.* 2004). The fatigue protocol was then conducted at one of either maximal (140Hz) or sub maximal (40Hz) stimulation frequency, and was composed of four distinct phases, the control phase, the treatment phase, the fatigue run, and the recovery phase, lasting a total of 130 minutes (four work loops at 10 minute intervals – Fig 5.3.1.). During the control phase (30 min) the muscle was incubated in standard Krebs-Henseleit solution and 3 assessments of muscle power

output were made in order to determine the stability of the preparation (Fig 5.3.1.). Immediately after the control phase, during the treatment phase (40 min), the circulating fluid was changed to Krebs-Henseleit solution containing 70µM caffeine and a further 3 assessments of muscle power output made (Fig 5.3.1.). 10 minutes later, whilst the muscle was still incubated in 70µM caffeine, the muscle was subjected to a fatigue run. Here all muscle preparations were subjected to 200 consecutive work loops at a cycle frequency of 5Hz using the same stimulation parameters as used in the rest of the protocol. A fatigue run of 200 work loops cycles was chosen in order to ensure that the muscle had sufficient time to fatigue until no net positive work was produced. Regardless of when fatigue occurred, all muscles were subjected equally to 200 consecutive loops. Endurance was defined as the time until each muscle preparation failed to produce net positive work. Subsequently muscle power output was recorded for every second loop until the muscle produced net negative work (i.e. work produced during shortening was less than the work required to re-lengthen the muscle), consequently statistical analysis for endurance uses this data (Figure 5.4.1). Forty minutes of caffeine treatment was allowed prior to the fatigue run in order to allow sufficient time for each preparation to reach its peak response to the caffeine treatment (James *et al.* 2005; Tallis *et al.* 2012; chapter 4). Typical work loop shapes at fixed time points during the fatigue run were plotted in order to make comparisons of the mechanical characteristics of the preparations between control and treatment muscle (Figure 5.4.3). The experiment concluded with 6 measurements (60 min) of power output in standard Krebs in order to monitor recovery of the muscle preparation post fatigue (Figure 5.4.3). In order to provide an experimental control the same protocol was followed, however, the muscle was incubated in standard Krebs-Henseleit throughout this recovery period. During the fatigue protocol each muscle preparation was used in only one experimental condition (control or treatment) and was assessed at only one stimulation frequency (140Hz or 40Hz) (32 muscles in total; n = 8 in each case).

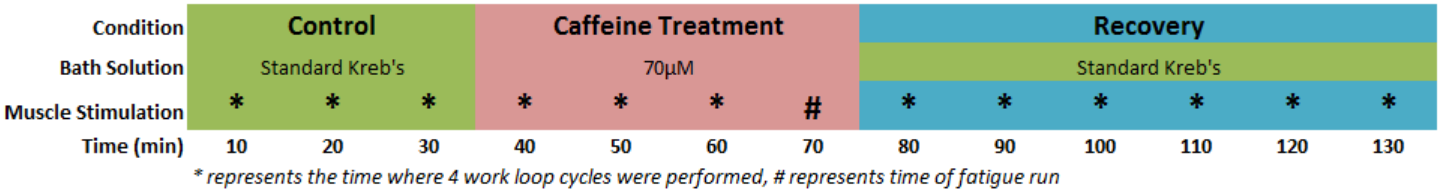


Figure 5.3.1. - Schematic of the work loop protocol to examine the ergogenic effect of 70µM caffeine on the fatigability of maximally and sub maximally fatigued mouse Soleus muscle

At the end of the experiment the muscle was detached from the rig, tendons removed, then weighed in order to calculate isometric stress ($kN.m^{-2}$) and normalised muscle power ($W.kg^{-1}$).

Statistical Analysis of the Data

Single factor analysis of variance (ANOVA) was performed in SPSS (Version 16, SPSS inc., IL, USA) in order to investigate the effect of stimulation frequency on isometric stress and work loop power in all muscle preparations used in the study. Single factor ANOVA with Tukey post hoc tests were used to test for differences in pre-treatment isometric stress and work loop power output between each experimental group (Pre-treatment muscle stress and power output for 140Hz and 40Hz were compared between muscles to be used in the control protocol and muscles to be used in the treatment protocol).

A two factor ANOVA was used to determine if the fatigue run induced a significant reduction in muscle power output over time and to assess whether this effect was significantly different between treatment groups. Tukey post hoc tests were conducted in order to assess whether endurance was significantly different between; 140Hz and 40Hz controls; 140Hz control and 140Hz caffeine; 40Hz control and 40Hz caffeine. As caffeine treatment was shown to have a significant effect on endurance, two-sample t-tests were conducted (Microsoft Excel 2007 version) at each time point for the same stimulation frequency in order to highlight any specific statistical difference in power output between control and caffeine treated

muscles at the same stimulation frequency (i.e. 2 t-tests at each time point; 40Hz control compared with 40Hz caffeine treated; 140Hz control compared with 140Hz caffeine treated).

A two factor ANOVA with Tukey post hoc tests was conducted on the data post the fatigue run in order to test for significant differences in the muscle's ability to recover from fatigue over the 60-minute duration and to test whether recovery differed between treatment categories. Therefore, treatment and time were used as the fixed factors and percentage of maximal power output the dependant variable. As before, two sample t-tests were conducted at each time point in order to assess specific differences in power output between control and caffeine treatments at the same stimulation frequency.

Results were interpreted as significant when $p < 0.05$. Values are displayed as mean ± standard error.

5.4. Results

The mean untreated maximal isometric tetanic stress and maximal work loop power for the entire population of soleus muscles used in the present study was 202 ± 11 kN m^{-2} and 33.1 ± 2.2 W kg^{-1} respectively, and was not significantly different to soleus muscle in Chapter 4 (Tukey $p>0.05$ in both cases).Reducing stimulation frequency from 140 to 40 Hz resulted in a significant reduction in both isometric stress (by 26%) and work loop power output (by 51%) in mouse soleus muscle (Table 5.4.1; ANOVA $p<0.001$ in both cases). Mean pre-treatment isometric stress and work loop power output at 140Hz were not significantly different between muscles to be used in the control protocol and muscles to be used in the caffeine treatment protocol (202 ± 15 kN m^{-2} and 32.4 ± 2.1W kg^{-1} in control, compared to 202 ± 18 kN m^{-2} and 33.8 ± 2.3 W kg^{-1} in caffeine treated, for stress and power respectively: Tukey $p>0.9$ in both cases). Similarly, there was no significant difference in sub-maximal stress or power output between muscles to be used in the control protocol and muscles to be used in the caffeine treatment protocol when tested at 40Hz (154 ± 20 kN m^{-2} and 15.9 ± 1.9 W kg^{-1} in control, compared to 146 ± 13 kN m^{-2} and 16.6 ± 1.4 W kg^{-1} in caffeine treated, for stress and power respectively: Tukey $p>0.95$ in both cases). This confirms that the muscles were of similar quality prior to treatment, of similar quality to previous studies (James *et al.* 2004; Tallis *et al.* 2012; chapter 4), and that any subsequent effects were solely due to caffeine.

Table 5.4.1. The effects of stimulation frequency on maximum isometric tetanus stress (force normalised to muscle cross-sectional area) and work loop power output (power output normalised to muscle mass) in mouse soleus muscle (n=32 pooled data pre allocation of preparations to control or caffeine treatment)

Twitch Stress ($kN.m^{-2}$)	41.9±3.9	
	*	*
Stimulation frequency	Tetanus Stress ($kN.m^{-2}$)	Net Max Work Loop PO ($W.kg^{-1}$)
40Hz	150±17	16±2
70Hz	188±17	24±2
140Hz	202±11	33±2

*[Data represented as Mean ± SE; * represent significant differences between stimulation frequencies]*

Effects of Stimulation Frequency and 70µM Caffeine Treatment on Endurance

For all treatments, the fatigue protocol elicited a significant decrease in active net power output of soleus muscle over time (Figure 5.4.1; ANOVA $p< 0.001$). Time to fatigue was significantly affected by treatment group (Figure 5.4.1; ANOVA $p<0.001$). In control muscle, 140Hz stimulation frequency resulted in significantly decreased endurance compared to 40Hz (Figure 5.4.1; Tukey $p=0.003$). Control muscle stimulated at 40Hz stimulation frequency, maintained power output above 80% of maximum for nearly twice as long as controls at 140Hz stimulation frequency.

After examination of individual time points, 140Hz control muscle produced significantly greater power than 140Hz caffeine treated from 3.6 seconds of the fatigue run onwards (Figure 5.4.1; two-sample t-test, d.f. = 14, $p< 0.05$ in each case). Treatment with 70µM caffeine caused a significant decrease in mean endurance (by 17.6%) compared to controls when soleus muscle was stimulated maximally at 140Hz (Figure 5.4.1; Tukey $p=0.001$). Mean net muscle power output was significantly higher for controls in work loops from 3.6s until negative work (Figure 5.4.1; two-sample t-test, d.f. = 14, $p<0.05$, in each case after

measurement at every second loop). Soleus muscle treated with 70µM caffeine when stimulated sub maximally demonstrated a significant mean increase (by 19.2%) in endurance compared to controls (Figure 5.4.1; Tukey p<0.001). Despite this no significant difference in mean muscle power output was found between 40Hz control and 40Hz caffeine treated at any individual time point throughout the fatigue run (Figure 5.4.1; two-sample t-test, d.f. = 14, *p*> 0.2 in each case). An increase in standard error over time represents an increase in individual variation in the rate of fatigue. However, in the 140Hz caffeine trial standard error remains low throughout fatigue, thus suggesting that the response to caffeine treatment varies little between individuals.

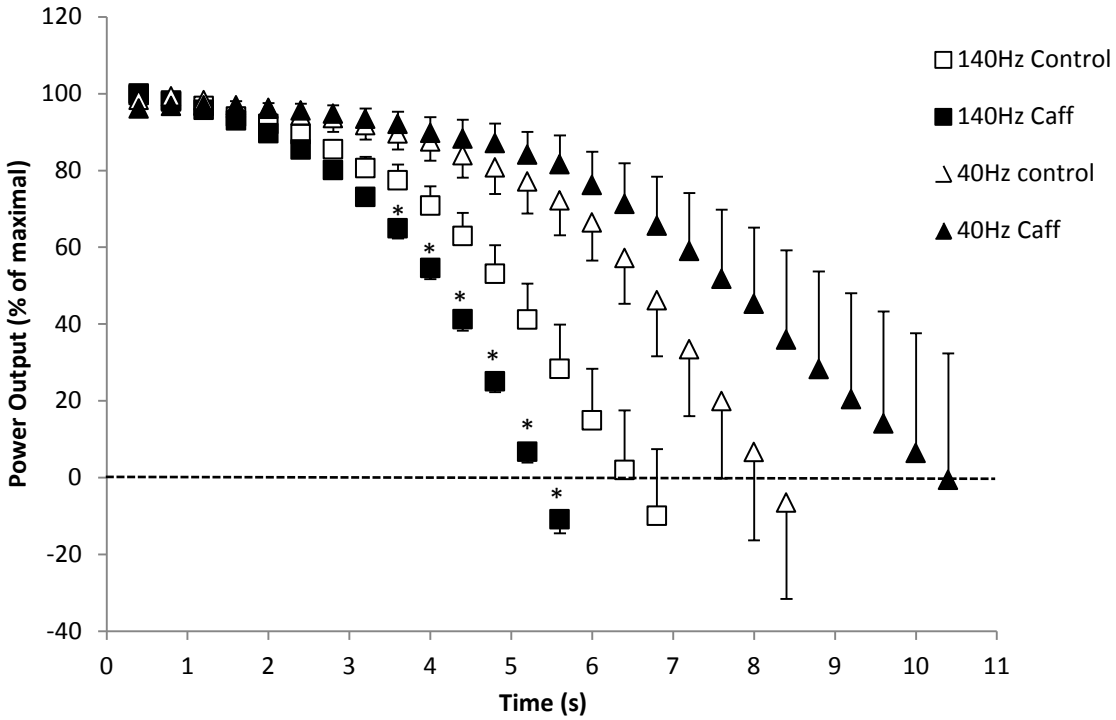


Fig.5.4.1 The effects of 70µM caffeine treatment and stimulation frequency on the rate of fatigue in mouse soleus muscle. Values are displayed as a percentage of maximal work loop power output [Data represented as mean & SE; n = 8 in each case; * represent significant differences in power output at each individual time point between 140Hz Control and 140Hz Caffeine treated]

There was very little variation in cumulative work between maximally stimulated control and caffeine treated muscles. Although power was slightly reduced in 40Hz caffeine treated muscle compared to control, the total cumulative work in soleus muscle treated with caffeine at 40Hz was 10% higher than in controls. It should be noted that the increased muscle power output demonstrated in control muscles compared to those treated with caffeine at 40Hz, should not be attributed to a caffeine effect. For example caffeine may still be acting to cause a significant increase in at 40Hz compared to controls, however generally the mean power output of control muscles is higher. The acute effect of caffeine at 140Hz and 40Hz has been previously discussed in chapter 4.

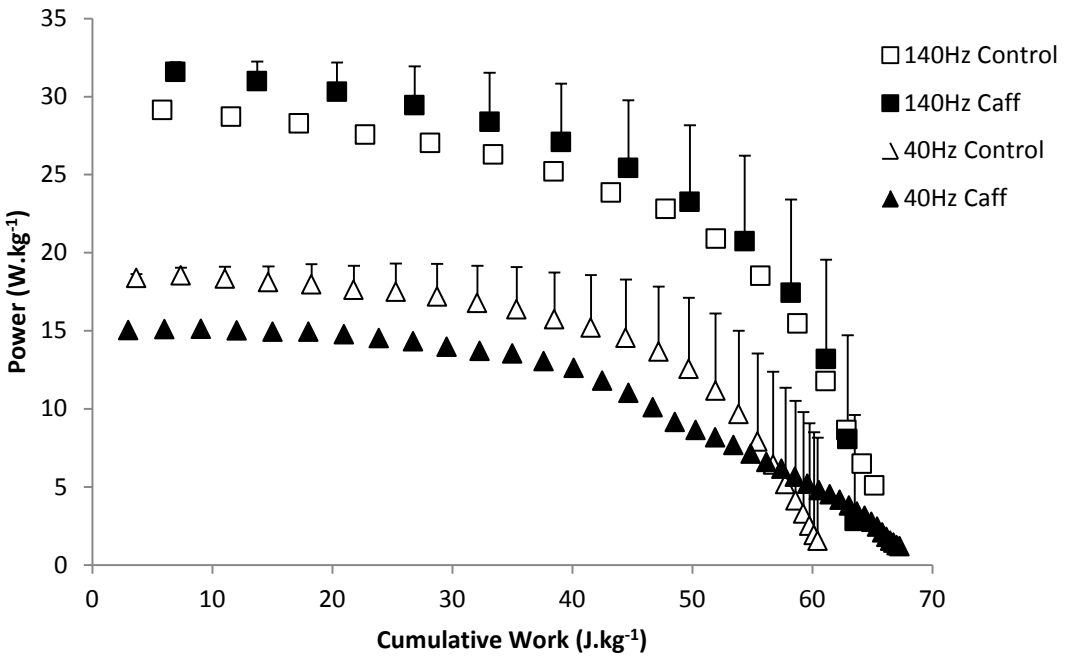


Fig.5.4.2 The effects of 70µM caffeine treatment and stimulation frequency on changes in the relationship between power output andcumulative work in mouse soleus muscle. [Data represented as mean & SE for every second loop of the fatigue protocol; n = 8 in each case] Note, that for clarity, not all error bars have been added.

The Effects of 70µM Caffeine on Work Loop Shape

The area of the work loop represents the net work of that particular cycle; the typical work loop shapes presented in Figure 5.4.3 clearly demonstrate that initial net work is larger at 140Hz stimulation frequency (Figure 5.4.3; A) compared to 40Hz stimulation frequency (Figure 5.4.3; C). At 140Hz stimulation frequency there is a significant reduction in peak and sustained force through shortening in the caffeine treated muscle (Figure 5.4.3; B) compared to controls (Figure 5.4.3; A). The shape of the loop 2.4 seconds after that start of fatigue indicates that there is an increase in relaxation time that is more pronounced in the caffeine trial (Figure 5.4.3; B) compared to controls (Figure 5.4.3; A). There is little difference in work loop shapes between controls (Figure 5.4.3; C) and those treated with 70µM caffeine (Figure 5.4.3; D) at 40Hz stimulation frequency. However the net work throughout fatigue appears to be greater in the caffeine trial compared to controls.

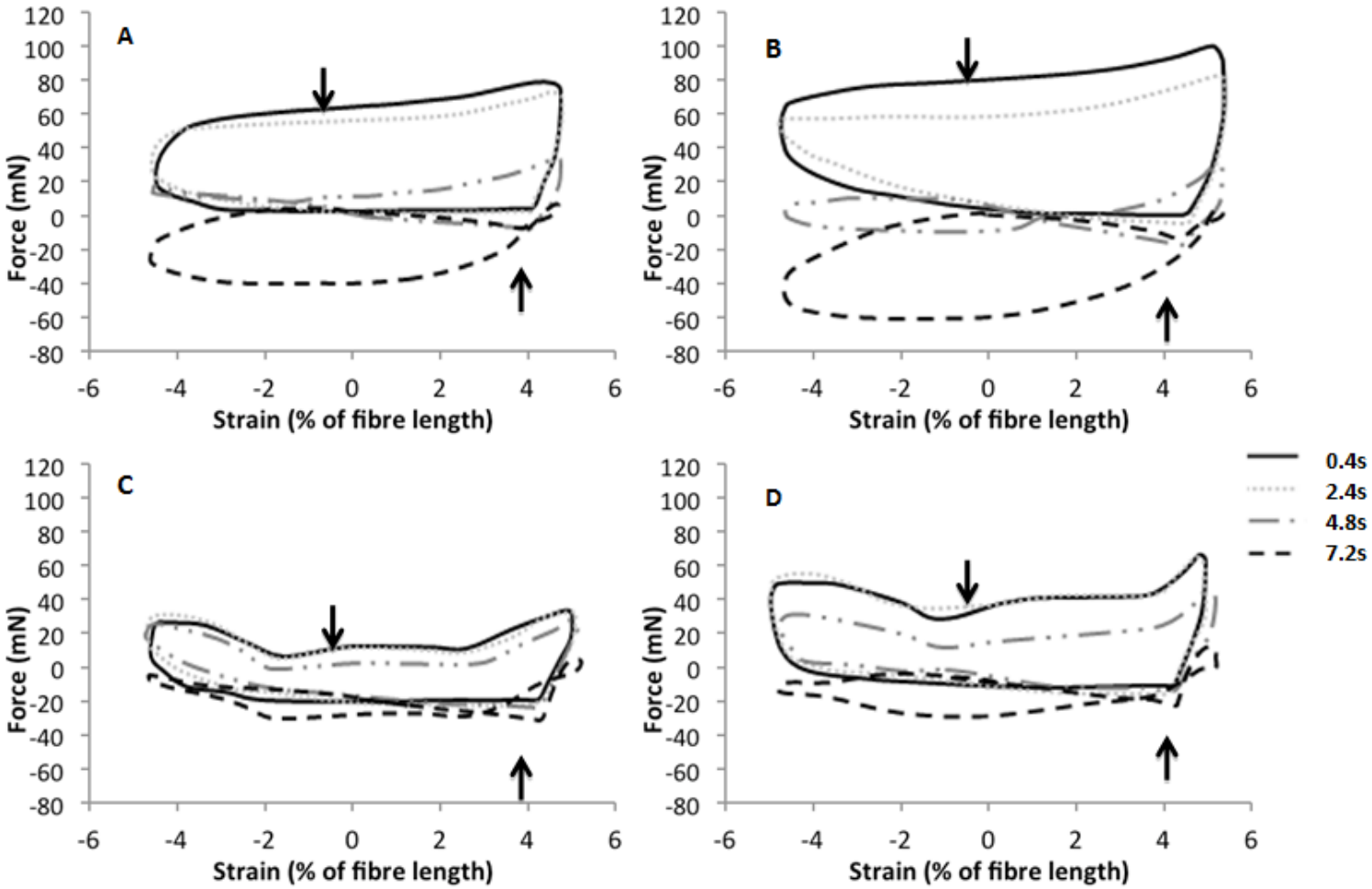


Fig. 5.4.3. Typical effects of fatigue on work loop shape for control soleus stimulated at 140Hz and 40Hz (A & C respectively) compared with those treated with 70µM caffeine (B 140Hz caffeine and D 40Hz caffeine) [Arrows indicate where stimulation typically started, towards the end of lengthening, and finished, during shortening; 0.4s, 2.4s, 4.8s, & 7.2s represent time since the start of the fatigue protocol]

Treatment Effects on Recovery from Fatigue

Soleus muscle fatigued at 140Hz in control Krebs recovered significantly better than the soleus muscle stimulated at 140Hz and fatigued in caffeinated Krebs (89% maximum after 30 minutes Vs. 63% maximum after 50 minutes; Figure 5.4.3; Tukey $p<0.001$). Muscle power output was significantly greater in control treatment at time points 20-60 minutes (Figure 5.4.3; two-sample t-test, d.f. = 14, $p>0.004$ in each case). There was no significant difference in recovery between the control (75% maximum after 30 minutes) and

those muscles fatigued in caffeine (71% maximum after 50 minutes) at 40Hz (Figure 5.4.3; Tukey p= 0.795). Control muscles stimulated throughout at 140Hz recovered significantly better, relative to pre fatigue maximum, than those stimulated throughout at 40Hz (Figure 5.4.3; Tukey p<0.001).

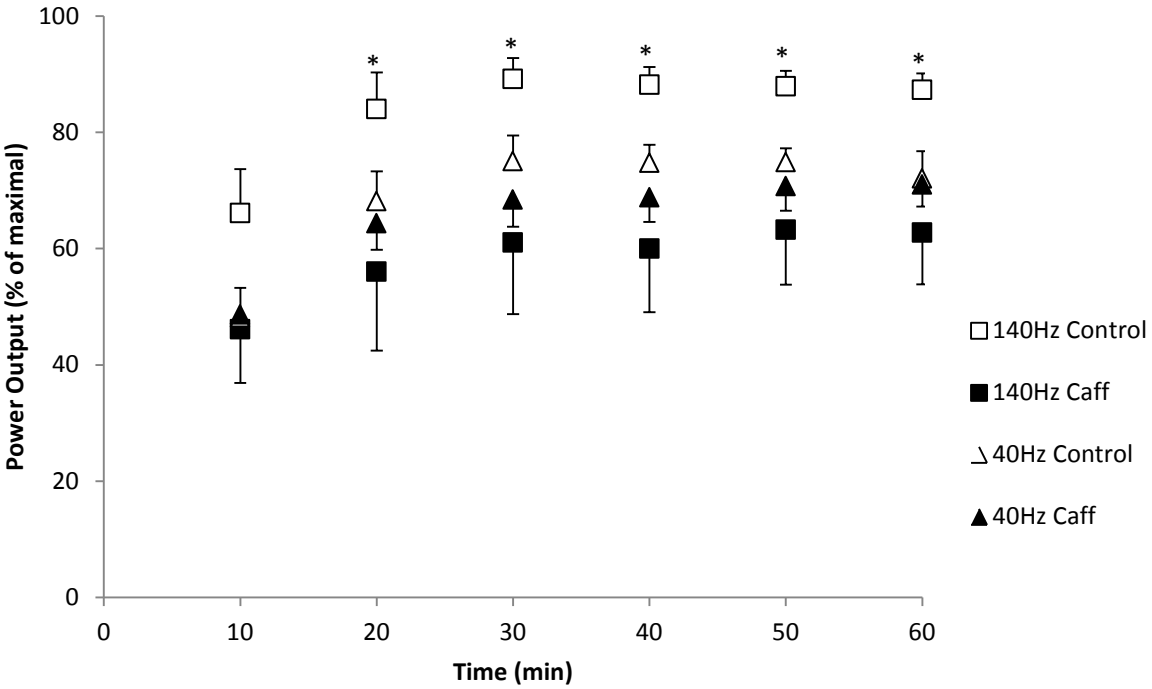


Fig. 5.4.4. Effect of 70µM caffeine treatment and stimulation frequency on recovery of soleus muscle following fatigue [Time 0 represents the end of the fatigue run; Data represented as mean & SE; n = 8 in each case; * represent significant differences in power output at each individual time point between 140Hz Control and 140Hz Caffeine treated]

5.5. Discussion

The mean untreated maximal isometric tetanic stress and maximal work loop power for the entire population of soleus muscles used in the present study was 202 ± 11 kN m^{-2} and 33.1 ± 2.2 W kg^{-1} respectively (Table 5.4.1), comparable to that previously reported for mouse soleus muscle (James *et al.* 2004; Askew *et al.* 1997; James *et al.* 1995; Vassilakos *et al.* 2009).Any potential variation in stress and power output between published studies could be attributed to muscle fiber type differences due to variation in strain and age of mice used and the environmental conditions at which they were kept.

The Effect of 70μM Caffeine Treatment on Endurance in Maximally Stimulated Muscle

Incubating soleus muscle in 70μM caffeine resulted in significantly shorter endurance (by 17.6%) at maximal (140Hz) stimulation frequency when compared to controls (Figure 5.4.1). Germinario *et al.* (2004) reported a faster decrease in isolated mouse soleus force when stimulated via repeated tetani after treatment with 2-5 millimolar caffeine (toxic to humans). The present results contrast those of James *et al.* (2005) who reported that 70μM caffeine failed to elicit any significant change in the endurance of maximally stimulated EDL. This suggests a fiber type specific effect of physiologically relevant concentrations of caffeine on muscular fatigue.

Skeletal muscle fatigue is associated with impaired SR Ca^{2+} release and reduced intramuscular Ca^{2+} concentration that is correlated with a reduction in muscle force (Allen *et al.* 1989; Allen *et al.* 2007). In relation to this, the fatigue induced increase in muscle relaxation time has also been attributed to reduced SR Ca^{2+} pump activity and an inability to decrease intramuscular Ca^{2+} between stimuli (Allen *et al.* 1989; Fitts 1994; Askew *et al.* 1997). An earlier study by Askew *et al.* (1997), using the work loop technique, demonstrated a fatigue induced increase in soleus muscle relaxation time. An increased relaxation time significantly accelerates fatigue when using the work loop technique by increasing the muscle's resistance to stretch and elevating the proportion of negative work per cycle [Askew *et al.* 1997; compare Figure 5.4.3B to 2A].

As previously discussed caffeine has been shown to augment excitation-contraction coupling, primarily by altering intramuscular Ca^{2+} handling (Magkos & Kavouras, 2005). The demonstrated combined effects of greater release of Ca^{2+} into the intramuscular space, increased myofibrillar Ca^{2+} sensitivity, slowing of the SR Ca^{2+} pump, and increased SR Ca^{2+} permeability, significantly modify skeletal muscle performance (Allen *et al.* 1989; Westerblad & Allen 1991; Allen & Westerblad 1995). The consequential decrease in rate of Ca^{2+} efflux from the intracellular space, due to the reduced action of the SR Ca^{2+} pump, is attributed to the caffeine induced increase in muscle relaxation time (Allen *et al.* 1989; Westerblad & Allen 1991).

In light of our previous findings (Tallis *et al.* 2012; chapter 4), it is likely that the caffeine treatment in the present study is having a muscle fiber type specific (when compared with EDL in the previous study by James *et al.* 2005) and activity level specific (i.e. only affecting maximally activated muscle) effect on relaxation rate and consequently power output during fatigue. Net power output attained via the work loop technique is the sum of the shortening work minus the energy required to elongate the muscle (Proske & Morgan 2001). If still active at the end of shortening a greater amount of energy is required to elongate the muscle as lengthening occurs against a greater resistance. The further elevated increase in relaxation time in caffeine treated, maximally activated soleus muscle, and the accumulation of this effect over time, is likely to result in a greater degree of muscle activation at the end of shortening. Consequently, in maximally activated muscles, the proportion of negative work per work loop cycle will be greater than in controls causing the muscle to fatigue faster. This is supported via examination of the work loop shapes. The later work loops in the fatigue run suggest that soleus muscle is failing to relax completely before re-lengthening when maximally stimulated, especially when treated with caffeine (Figure 5.4.3 loops 2.4s and 4.8s). The fact that a similar mechanism was not prevalent in EDL (James *et al.* 2005) highlights the enhanced ability of a faster muscle phenotype to remove calcium form the intramuscular space.

With reference to the cumulative work produced in maximally fatigued muscle, although the caffeine treated muscle is fatigue more quickly, the total amount of work done by the muscles is the same. This

therefore indicates that the caffeine treated muscles were producing greater power output per cycle compared to controls, in agreement with the findings of Tallis *et al.* (2012; Chapter 4).

The Effect of 70μM Caffeine Treatment on Endurance in Sub Maximally Stimulated Muscle

Treatment of soleus with 70μM caffeine resulted in a mean significant increase in endurance in sub maximally stimulated soleus muscle (by 19.2%) compared to controls (Figure 5.4.1). In addition a 10% increase in the cumulative work over the period of fatigue was found (figure 5.4.2). Producing a greater amount of power, or applying that power over a greater duration (as demonstrated in figure 5.4.1) will contribute to this phenomenon. In contrast to all previous literature (James *et al.* 2005; Germinario *et al.* 2004; Reading *et al.* 2004) the present results are the first to indicate a caffeine induced direct increase in skeletal muscle endurance. In this instance, it is difficult to make a direct comparison to previous findings primarily due to differences in the method used and the previously high concentrations of caffeine being significantly greater than the physiologically relevant dose (Germinario *et al.* 2004; Reading *et al.* 2004). Most previous studies have induced fatigue via repeated isometric tetani, however, this would be a poor predictor of fatigue induced by the patterns of cyclical muscle length changes used *in vivo* as in isometric studies the muscle is held at constant; therefore changes in the force-velocity, length-force and passive properties of the muscle are not considered (Josephson 1985). The present work is the first to indicate a caffeine induced, direct increase in skeletal muscle endurance, and further suggests a complex muscle specific caffeine response affected by the intensity of muscle activity.

Examination of the work loop shapes (Figure 5.4.3) indicates that, in contrast to maximally activated muscle, there appears to be no difference in the ability of the muscle to relax in the caffeine treated muscle (Figure 5.4.3;D) when compared to controls (Figure 5.4.3;C). At this reduced stimulation frequency, the interval between individual stimuli will be increased allowing a greater duration for the reduction in intramuscular Ca^{2+} between successive stimuli. Therefore, in this instance, it is probable that fatigue is less affected by an increased relaxation time and largely results from a decline in force. As with previous research (James *et al.* 2005; Tallis *et al.* 2012; chapter 4), it is likely that the increase in time to

fatigue is attributed to the direct effect of caffeine to increase force via augmented Ca^{2+} release without the prevalent effects of amplified negative work observed in maximally activated caffeine treated muscle. With the muscle stimulated at 40Hz, significantly below the 140Hz required for maximal activation, any caffeine induced increase in intramuscular Ca^{2+} is likely to still be well within the calcium handling capabilities of the SR Ca^{2+} pump. Subsequently, unlike with maximally activated muscle, the level of activation at the end of work loop shortening is unlikely to vary between control and caffeine treated muscles thus limiting the occurrence of increased negative work observed in maximally activated soleus muscle.

Recovery from Fatigue Following Treatment with 70μM Caffeine

Fatigue of maximally stimulated (140Hz) soleus muscle treated with caffeine resulted in a significant reduction in recovery of net power (by 26%) compared to controls (Figure 5.4.4). The previously highlighted caffeine induced increase in negative work during fatigue at this intensity is the likely cause of this, as elevated eccentric activity is associated with increased muscle damage (Dickinson *et al.* 2000). Damage brought about by eccentric exercise would result in a decrease in the ability to recover after fatigue, and is an effect previously observed by James *et al.* (2005) using 10mM caffeine treatment on mouse EDL and soleus. At submaximal, 40Hz, stimulation frequency there was no significant difference between mean recovery of controls and those fatigued in caffeine (Figure 5.4.4).

Practical Implications of the Present Findings

The present findings are the first to investigate the effects of physiological caffeine concentration on the fatigability of predominantly slow twitch muscle and demonstrate decreased endurance when maximally stimulated, and prolonged endurance when sub maximally stimulated. This, in conjunction with our earlier findings in predominantly fast twitch EDL (James *et al.* 2005), indicates a muscle specific caffeine fatigue response which is further related to the activation level of the muscle.

Tallis *et al.* (2012; chapter 4) further demonstrated a potentiation in acute muscle power output, independent of stimulation frequency, that was greater in mouse soleus muscle (up to 6%) compared to EDL (up to 3%). If applied uniformly across the fatigue data, a 6% potentiation would result in a slight upwards shift in the fatigue curve, but is unlikely to significantly affect the overall findings.

Although the sinusoidal length change pattern used in the current study provides an approximation of *in vivo* cyclical muscle activities, it is a simplification of the length change waveforms used in real life locomotion (Dickinson *et al.* 2000). *In vivo* the pattern of fiber stimulation and length change waveforms are likely to be manipulated throughout movement (Wakeling & Rozitis 2005). Therefore if a muscle is producing too much eccentric force the duration of stimulation is likely to be reduced in order to lessen any associated muscle damage. Subsequently, *in vivo,* the activation of caffeine treated fibers may be reduced to produce the same power as controls, with the activation of fewer muscle fibers, thus prolonging endurance. However, these proposed differences between our experimental protocols and what may happen *in vivo* are unlikely to affect the overall findings of this study.

Conclusion

The direct treatment of mouse soleus muscle with 70µM caffeine resulted in a stimulation frequency specific effect on endurance. When fatigued at a stimulation frequency that elicited maximal force (140Hz), soleus treated with caffeine fatigued significantly faster, which may relate to a caffeine induced increase in muscle relaxation time. However, when the same experiment was conducted on soleus muscle stimulated at a low stimulation frequency (40Hz) that yielded a submaximal force, caffeine treatment resulted in a significant increase in time to fatigue. The present findings indicate a complex response between activation intensity and the effect of caffeine that should probably be examined over a greater range of stimulation frequencies. The current findings highlight an enhanced ergogenic benefit of caffeine in prolonged sub maximal activities that are primarily powered by slower muscle fibers. This extends our earlier work (Tallis *et al.* 2012; chapter 4), by being the first to demonstrate a stimulation frequency dependent response of endurance to physiological concentrations of caffeine.

6. Does a Physiological Concentration of Taurine Increase Acute Muscle Power Output, Time to Fatigue, and Recovery in Mouse Soleus (Slow) Muscle With or Without the Presence of Caffeine?

6.1 Abstract

The value of energy drinks in providing ergogenic benefit across a range of sporting activities has been firmly established. It is widely recognised that caffeine, a constitute of many energy drinks, can promote performance enhancing effects on endurance, strength, and power activities. Recently, physiological concentrations of caffeine have been shown to directly affect skeletal muscle performance (Tallis *et al.* 2012; chapter 4). Additionally, taurine is also found in high quantities in many energy drinks, although the benefit of acute ingestion of taurine is less well understood. *In vitro* models have demonstrated that supraphysiological caffeine induced contracture can be further potentiated when combined with high concentrations of taurine. The present study used isolated mouse soleus muscle to investigate the effects of physiological concentrations of taurine alone and taurine and caffeine combined on: 1) acute muscle power output; 2) time to fatigue; and 3) recovery from fatigue. Treatment of mouse soleus muscle with taurine failed to elicit any significant difference in any of these parameters when compared to controls. Treatment with taurine and caffeine combined resulted in a significant increase in acute muscle power output and faster time to fatigue. The ergogenic benefit posed by taurine and caffeine combined was not different from the effects of caffeine alone. In conclusion, an acute muscle taurine treatment, which could be achieved via consumption of energy drinks, does not directly improve skeletal muscle performance.

Key Words: Work Loop, Force, Skeletal Muscle, Endurance, Ergogenic Aid

6.2. Introduction

The consumption of energy drinks to provide an ergogenic benefit in psychological and physical performance is widely recognised and common practice for many athletes (Alford *et al.* 2001; Graham *et al.* 2001, Davis & Green, 2009; Duncan & Oxford, 2011). The carbohydrate content of such products provides the addition fuel for energy production; in addition a number of supplementary ingredients are included as mechanists for other performance enhancing reactions. The ergogenic effect of energy drinks is largely related to the presence of caffeine; however the scientific understanding of other ingredients in their ability to enhance sporting performance has received far less attention. In addition to high concentrations of caffeine and various sugars, the amino acid taurine (Amino ethane Sulfonic Acid) is a constituent of many energy drinks and is believed to contribute to the performance enhancing effect (Geib *et al.* 1994). Therefore, the present study aims to assess the benefit of acute taurine supplementation to promote a direct enhancement of muscle performance.

Taurine is readily synthesized by the body and is found in high concentrations in mammalian tissue particularly the heart and skeletal muscle (Schaffer, 2010). Although the mechanism of action are unclear it has been established that taurine affects a number of electro physiological and biochemical parameters, with its greatest functions relating to osmoregulation, regulation of oxidative stress, and cell signalling (Cuisinier *et al.* 2002; Matsuzaki*et al.* 2002; Oja & Saransaari, 2007; Scaffer *et al.* 2010). Renal uptake of taurine is low and hence taurine is the major urinary amino acid with diet dependant losses ranging from 65-250mg (Galloway *et al.* 2008). A clear role of taurine in maintaining mammalian myocardial and skeletal muscle contractile function has been demonstrated throughout previous research (Schaffer, 2010).Warskulat *et al.* (2004) used -/- mice to identify taurine as a crucial amino acid for the maintenance of total function and exercise capacity of skeletal muscle. The almost complete depletion of skeletal muscle taurine that resulted caused a reduction of total exercise capacity by more than 80%. Furthermore, reducing the intercellular concentration of taurine has commonly caused reductions in

skeletal muscle function, primarily resulting in a decrease in muscle force production (Hamilton *et al.* 2006).

In vivo and *in vitro* evidence strongly suggests that chronic taurine supplementation can positively enhance skeletal muscle performance (Imagawa *et al.* 2009). Chronic taurine supplementation in rats has been shown to lead to large increases in muscle taurine concentration and a consequential enhancement of muscle force. This in turn corresponds to an increase in muscle calsequestrin content indicating a greater Ca^{2+} SR concentration (Goodman *et al.* 2009).

The present study evaluates the skeletal muscle specific response to acute supplementation of taurine that may occur via one off consumption of energy drinks prior to sporting performance. From a whole body human perspective Geib *et al.* (1994) concluded that 'Red Bull' energy drink had a greater effect at increasing endurance if the taurine content was present. However, evidence discussing the one off effects of taurine supplementation on physiological performance is limited. *In vitro* high concentrations of taurine have been shown to increase isometric force in fast skeletal muscles of rat and slow abdominal extensor muscle of crayfish (Galler & Hutzler, 1990; Bakker & Berg 2002). The mechanism of action is believed to occur via modification of ion channel function and Ca^{2+} homeostasis (Galler & Hutzler, 1990; Bakker & Berg 2002; Camerino *et al.* 2004; Cuisiner *et al.* 2000).

There is little evidence examining the maximal one off dosage of taurine for human consumption and furthermore, research regarding the maximal blood plasma concentration is scarce. In humans taurine has been used in doses up to 6g per day and has been used intravenously in doses up to 5g without a toxic effect (Milei *et al.* 1992; Mizushima *et al.* 1996). Galloway *et al.* (2008) reported a peak blood plasma concentration of 750µM following oral ingestion of 1.66g of taurine. Based on the results of Galloway *et al.* (2008), the taurine concentration used in the present study (2.64mM) would compare to 5.8g taurine supplementation and thus represents a high, but physiologically relevant concentration.

The performance enhancing benefits of caffeine in human performance has been widely established in endurance (activity lasting greater than 30 minutes), power and strength activities (Graham, 2001; Davis & Green, 2009). James *et al.* (2005) and Tallis *et al.* (2012; 2013; chapter 4 & 5) used mouse EDL (relatively fast) and soleus (relatively slow) isolations to demonstrate the direct effect of physiologically maximal concentrations of caffeine (70μM) on acute muscle power output and time to fatigue. These studies indicated a potentiation of acute muscle power output across maximal and submaximal intensities with a greater response in soleus muscle compared to EDL. Caffeine treatment had various effects on time to fatigue depending on muscle fiber type and the intensity of stimulation. This caffeine induced muscle force potentiation has been shown to occur via direct adenosine receptor antagonism resulting in alterations in excitation contraction coupling primarily due to changes in intramuscular Ca^{2+} handling (Daminai *et al.* 1996; Bhat, 1997; Fredholm *et al.* 1997; Rossi *et al.* 2001; Davis & Green, 2009; Magkos &Kavouras, 2005). A limited number of diverse studies examining skinned rat heart to whole body human performance have demonstrated that taurine, when combined with caffeine, could result in further potentiation of the effects of caffeine alone (Geib *et al.* 1994; Steele *et al.* 1990). However, this effect has never been established using physiologically relevant concentrations of taurine and caffeine directly on skeletal muscle. With the caffeine induced increase in Ca^{2+}release and the proposed increase in Ca^{2+}myofilament sensitivity a combination of treatments may produce a greater ergogenic benefit.

Although verified to a greater extent in predominantly fast muscle, a reduction in taurine concentration with prolonged muscle activation has been identified in all skeletal muscle fiber types (Kim *et al.* 1986; Matsuzaki *et al.* 2002). The present study will aim to investigate whether a physiological dose of taurine can reduce the rate of muscle force deterioration. The time to fatigue will also be assessed with taurine and caffeine combined to establish if the effect is greater than caffeine treatment alone. Further to this the effects of increasing the concentration of taurine following fatigue will be examined in order to investigate whether the intracellular concentration can be restored and a consequential increase in the efficiency of recovery induced. The present study will also assess the effects of physiologically relevant concentrations of caffeine (70μM) on soleus muscle recovery.

The aims of the present study are to use isolated mouse soleus muscle to assess the direct effects of physiologically relevant concentrations of taurine alone (2.64mM), caffeine alone (70µM), and taurine and caffeine combined on: 1) acute maximal muscle power output; 2) time to fatigue; 3) recovery following fatigue. The information regarding the effects of caffeine alone is derived from previous publications by Tallis *et al.* (2012, 2013; chapter 4 & 5) and in this instance the data will be used solely for comparison. Normal plasma concentration of taurine is 30-60 µmol/l which is significantly lower in relation to an intramuscular content between 50-60 mmol/kg (Galloway *et al.* 2008). Subsequently it is suggested that an acute, direct taurine concentration equivalent to a typical dose from energy drinks, will not be great enough to cause significant improvements in the power output, endurance or recovery from fatigue of mouse soleus muscle. Furthermore when combined with 70µM caffeine, taurine treatment will show no further potentiation than the effect of caffeine alone.

6.3. Materials and Methods

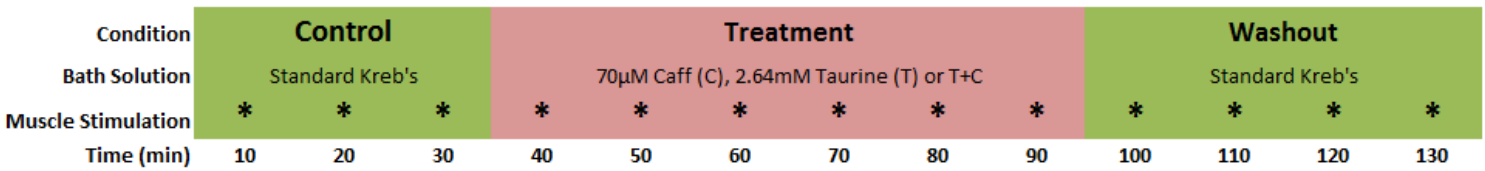
A more detailed account of the methods is given in the general methods section (chapter 3).

Soleus muscle was isolated from the right hind limb of 8-10 week old mice (body mass = 31.1±0.24 g, mean ± SE, *n* =72). Each muscle preparation was then placed in to the muscle bath that was continually circulated with Krebs-Henseleit solution at a constant temperature of 36 ± 0.02^{o}C. Initially muscle length and stimulation amplitude (12-16V) were optimised to elicit maximal isometric twitch force. Using these parameters and a burst duration of 320ms the muscle preparation was subjected to a series of isometric tetani. Stimulation frequency (usually 140Hz) was further optimised to evoke maximal tetanus force. To ensure sufficient recovery a 5-minute rest period was imposed between each tetanus.

Work loop experiments were conducted in order to assess the effects of taurine and taurine combined with caffeine on each of acute muscle power output, fatigability, and fatigue recovery. A concentration of 2.64 mM was used for taurine treatment; this was estimated from the maximal concentration available if the taurine from a single can of Red Bull (1g) was instantaneously absorbed and equally distributed throughout the blood plasma. The comparison to Red Bull has been made as it is a widely recognised energy drink with a taurine content representative of similar products available; therefore, testing this taurine concentration seemed ecologically valid. We recognise that the maximal concentration of taurine cannot be instantaneously absorbed and equally distributed following human ingestion; however it should be considered a high physiological concentration that could be derived via multiple consumption of such energy drinks. In order to test whether 2.64 mM of taurine alone (TAU) or 2.64 mM taurine combined with 70µM caffeine (maximal human physiological concentration) (TC) affected acute muscle power output, time to fatigue, or recovery from fatigue, each soleus muscle preparation was subjected to one of the following protocols. i.e. each muscle preparation was used to determine the effects of treatment on acute power output or time to fatigue or recovery from fatigue. 70µM is recognised as the human physiological maximum, the caffeine only treatment data at this concentration has been published previously (Tallis *et al.* 2012; 2013; chapter 4 & 5).

Acute Power Output

As in chapter 1 and 2, during work loop experiments soleus muscle power was measured at 5Hz cycle frequency using a strain of 0.10, and a fixed phase shift of -10ms. In order to test the acute effect of a given treatment the muscle was subjected to four work loop cycles at 10 minute intervals over 130 minutes (Fig 6.3.1.). During the initial three measurements the muscle was maintained in standard Krebs-Henseleit solution. The fluid was then changed to Krebs-Henseleit solution containing either 2.64 mM taurine (TAU) or 2.64 mM taurine combined with 70µM caffeine (TC). This experimental treatment was maintained for 60 minutes. Following this, the solution was then changed to standard Krebs-Henseleit solution, for a wash out period of 40 minutes, to establish if the effect of treatment was reversed, returning the muscle to its pre-treatment state (Fig 6.3.1.). The second work loop of four was always used as the indicative measure of muscle power output at each time point.



** represents the time where 4 work loop cycles were performed, # represents time of fatigue run*

Figure 6.3.1. - Schematic of the work loop protocol to examine the ergogenic effect of Taurine and caffeine on acute muscle power output of maximally stimulated mouse soleus muscle

Time to Fatigue

In order to test the effect of a given treatment on time to fatigue the muscle was again subjected to sets of four work loops at 10-minute intervals over a 130-minute duration. During the initial 3 measurements the muscle was maintained in standard Krebs-Henseleit solution. For the subsequent 40 minutes the fluid was changed to TAU or TC. The fourth measurement in the treatment solution was a fatigue run consisting of 100 consecutive work loop cycles; data was collected for every second loop. Forty minutes of incubation in the treatment solution was allowed prior to the fatigue run to give sufficient time to allow

each preparation to respond to the treatment (Tallis *et al.* 2012; 2013; chapter 4 & 5). Fatigue was determined to have occurred when the muscle preparation produced net negative work. Directly after the fatigue run the circulating fluid was changed back to standard Krebs-Henseleit solution and recovery was monitored for 60 minutes by subjecting the muscle to a set of four work loop cycles at 10 minute intervals over this duration. The control protocol followed the same process however the muscle was maintained in standard Krebs-Henseleit solution throughout.

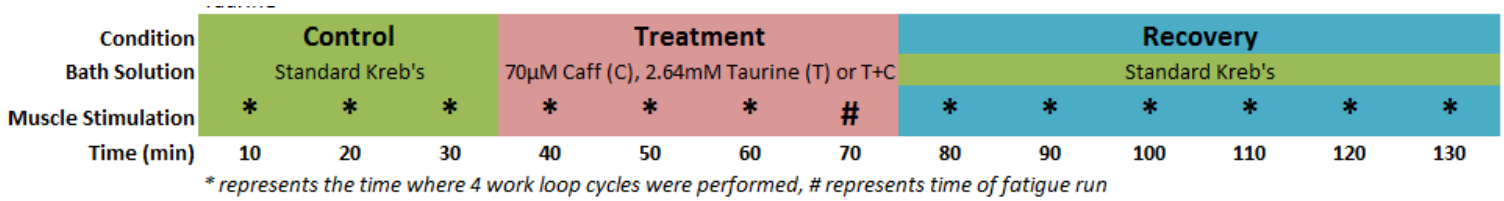


Figure 6.3.2. - Schematic of the work loop protocol to examine the ergogenic effect of Taurine and caffeine on mouse soleus muscle maximally stimulated to fatigue

Recovery from Fatigue

The protocol for testing the recovery from fatigue was the same as that used in the time fatigue protocol above, however, in the time up to and inclusive of the fatigue run the muscle was incubated in standard Krebs-Henseleit solution. Immediately after the fatigue run the solution was changed to TAU, TC or Caffeine (CAF) and recovery was monitored for the next hour (Fig 6.3.3).

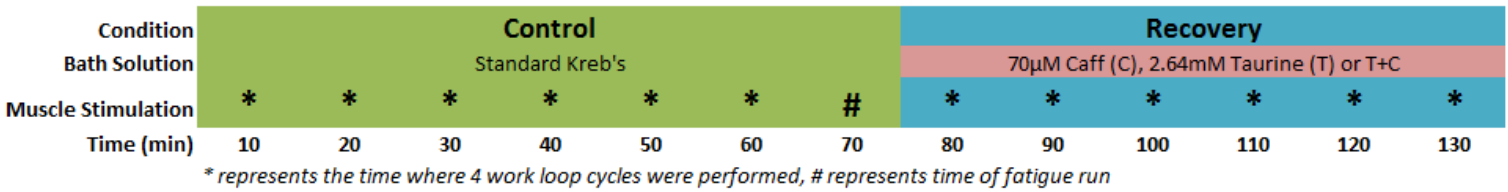


Figure 6.3.3. - Schematic of the work loop protocol to examine the ergogenic effect of Taurine and caffeine on mouse soleus muscle on recovery of maximal power output following maximally stimulated fatigue

At the end of the experiment the muscle was detached from the rig, tendons removed, then weighed in order to calculate isometric stress ($kN.m^{-2}$) and normalised muscle power ($W.kg^{1}$).

Statistical Analysis of Data

In order to compare the quality of muscle between treatment groups a single factor ANOVA was conducted on maximal untreated isometric stress and work loop power output data.

Due to the gradual development of an anoxic core in control conditions muscle power output will very gradually decrease over time (Barclay, 2005). For the muscles tested in the present study power output decreased to an average 96 ± 1% of initial power over the 130-minute duration of the work loop protocol. In order to avoid the deterioration in muscle performance masking the effects of treatment, a 1^{st} order regression equation was calculated using the pre-treatment control data and post treatment washout control data in order to identify the linear relationship between muscle power output and time. This regression equation was then used to determine theoretical control muscle power output for each time point during caffeine treatment (as in James *et al.* 2005; Tallis *et al.* 2012; chapter 4 & 5).

Initially, for the acute power output data, pre-treatment controls were compared against post treatment washout controls for each experimental group. There was no significant difference between this control data (Fig 6.4.1; paired t-test $p>0.6$ in all cases) therefore, it was assumed that any subsequent change in muscle power when incubated in TC, TAU or CAF was an effect of the given treatment. In order to assess the effects of treatment for each acute response the control data was compared directly against treatment data via paired t-tests. A single factor ANOVA was conducted in order to identify whether the given increase in power output was different between treatments (TC vs.TAU vs. CAF). Tukey post hoc tests were used to establish where these differences occurred.

A two factor ANOVA was used to determine whether muscle power output changed over time. A second two factor ANOVA was used to determine whether the time to fatigue differed between treatment groups. In each case Tukey post hoc tests were performed where applicable. Single factor ANOVA were

used at each time point in order to assess whether power output was significantly different between treatments at each time point of the fatigue run.

A two factor ANOVA was implemented to identify whether power output increased significantly over recovery time and whether recovery differed between treatment groups, Tukey post hoc tests were used for treatment. Single factor ANOVA were conducted at each time point in order to identify if muscle power output differed between treatments.

Results were interpreted as significant when $p < 0.05$. Values are displayed as mean ± standard error.

6.4. Results

The mean maximal isometric twitch and tetanus stress for soleus muscle (n=76) was 38.6 ± 1.5 kN $m^{-2}$and 219 ± 7 kN m^{-2}respectively. Mean untreated maximal muscle power output was 32.8 ± 1 W kg^{-1}(n=76). Untreated mean maximal isometric tetanus stress and work loop power output were not significantly different between experimental groups (single factor ANOVA main effect p>0.35 in both cases) or to that in chapters 4 and 5 (Tukey p>0.05 in both cases). Therefore, it is assumed that the muscles were of a similar quality between the experimental groups and that any subsequent differences in response occur due to the effects of treatment.

The Effect of Physiological Concentrations of Taurine and Caffeine on Muscle Power Output

Treatment of soleus muscle with caffeine alone or taurine combined with caffeine resulted in a significant increase in acute maximal muscle power output of 4.1% and 6.4% respectively (Fig 6.4.1; paired t-test p<0.03 in both cases). Treatment with taurine alone failed to elicit any significant increase in muscle power output (Fig 6.4.1; paired t-test p=0.118).

The treatment induced benefit was significantly dependant on treatment (Fig 6.4.1; single factor ANOVA main effect p=0.03). There was no significant difference between the benefit imposed by caffeine alone when compared with taurine and caffeine combined (Fig 6.4.1; Tukey p=0.628). The treatment effect of caffeine was significantly higher than that of taurine alone (Fig 6.4.1; Tukey p=0.027), however there was no significant difference between taurine and caffeine combined and taurine alone (Fig 6.4.1; Tukey p=0.178).

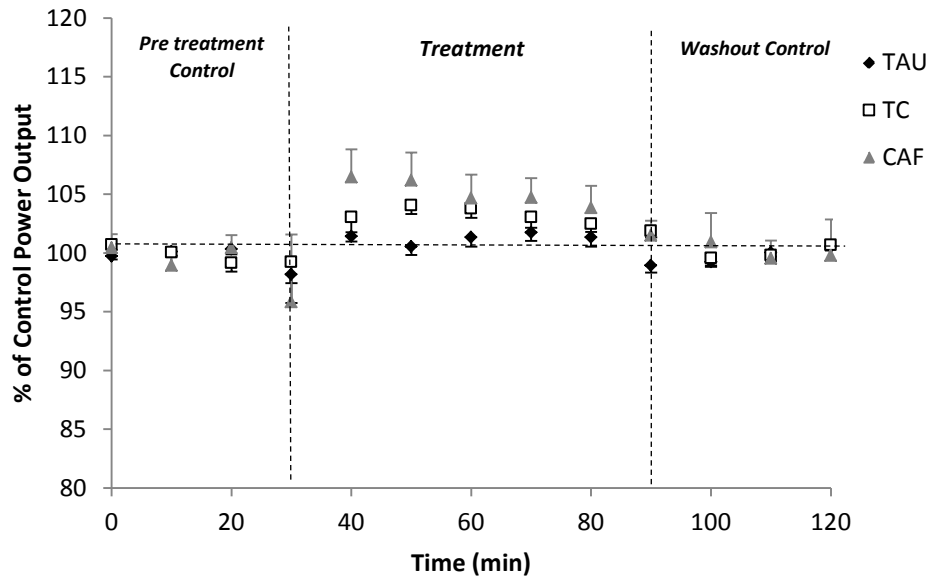


Figure 6.4.1. – The mean acute effect of physiological concentrations of taurine alone (TAU), caffeine alone (CAF), and taurine and caffeine combined (TC) on net work loop power output in maximally stimulated mouse soleus muscle [Data represented as mean & SE] n=10 for TAU and TC, n=8 for CAF.

The Effect of Physiological Concentrations of Taurine and Caffeine on Time to Fatigue

The fatigue protocol was effective in inducing soleus muscle fatigue as net power output reduced significantly over time (Fig 6.4.2; two factor ANOVA main effect of time $p<0.001$). There was a significant effect of treatment on time to fatigue (Fig 6.4.2; two factor ANOVA main effect $p<0.001$). Treatment of soleus muscle with caffeine alone or with taurine and caffeine combined resulted in a significantly decreased time to fatigue (time to net negative work increased by 30% and 15% respectively) compared to controls (Fig 6.4.2; Tukey $p<0.001$ in both cases). Soleus muscle treated with caffeine fatigued significantly faster than muscle treated with taurine and caffeine combined (Fig 6.4.2; Tukey $p<0.001$). There was no significant difference in time to fatigue between soleus muscles treated with taurine alone compared to controls (Fig 6.4.2; Tukey $p<0.001$).

Soleus muscle treated with caffeine produced significantly less normalised power compared with muscle treated with taurine from 3.2s onwards, from 3.6s onwards when compared with controls, and from 4.8s when compared with taurine and caffeine combined (Fig 6.4.2; Tukey$p<0.04$ in all cases). There was no significant difference in the mean net power output at each time point in any other treatments (Fig 6.4.2; single factor ANOVA Tukey$p>0.05$ in all cases).

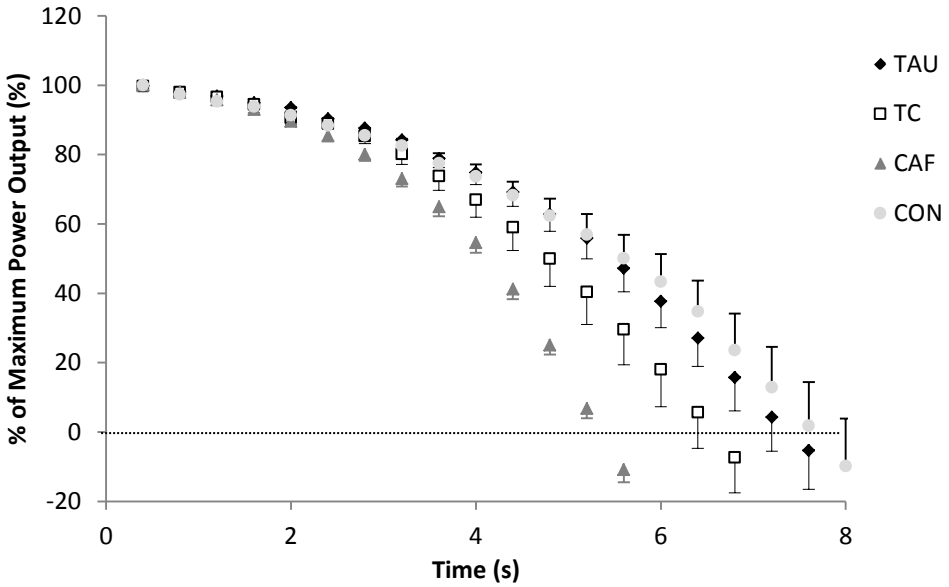


Figure 6.4.2. –The effects of physiological concentrations of taurine alone (TAU), caffeine alone (CAF), and taurine and caffeine combined (TC) on time to fatigue in maximally stimulated mouse soleus muscle. Values are displayed as a percentage of maximal net work loop power output. [Data represented as mean & SE] n=8 in each case.

When plotted as cumulative work, a similar pattern to that demonstrated with differences in time to fatigue is observed. Although initial power output was similar between muscles, caffeine treated muscle produced significantly reduced cumulative work compared to all other treatment groups (Fig. 6.4.3).

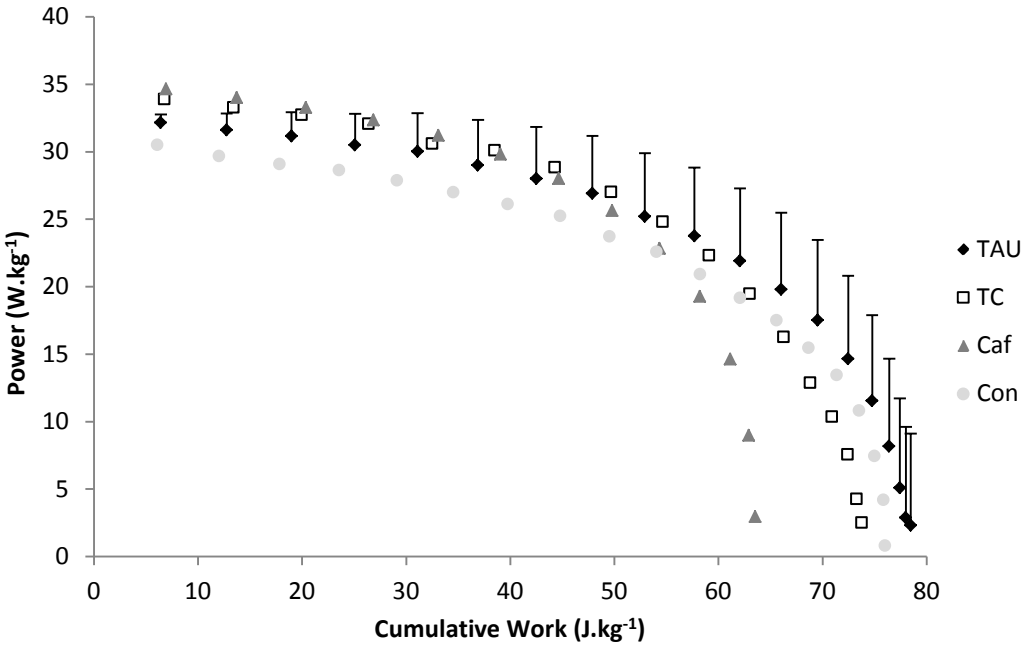


Figure 6.4.3. The effects of physiological concentrations of taurine alone (TAU), caffeine alone (CAF), and taurine and caffeine combined (TC) on the relationship between power output and cumulative work in mouse soleus muscle. [Data represented as mean & SE for every second loop of the fatigue protocol; n = 8 in each case] Note, that for clarity, not all error bars have been added.

The Effect of Physiological Concentrations of Taurine and Caffeine on Recovery from Fatigue

Net power output significantly increased over time during recovery from fatigue (Fig 6.4.4; two factor ANOVA main effect $p<0.001$). The level of soleus muscle recovery was significantly dependant on treatment (Fig 6.4.4; two factor ANOVA main effect $p=0.035$). Soleus muscle treated with caffeine had a mean peak recovery at 91.5% of the pre fatigue maximum; this was significantly better than the 80.8% recovery with taurine and caffeine combined (Fig 6.4.4; Tukey $p=0.031$). Controls and taurine alone recovered to 89.2% and 86.3% of pre fatigue maximum and there was no significant difference in recovery between any other treatments (Fig 6.4.4; Tukey $p>0.105$ in all cases). Despite highlighted differences in recovery between treatments the net power output was not significantly different between

treatments at any given time point during the recovery period (Fig 6.4.4; single factor ANOVA Tukey $p>0.156$ in all cases). For all treatments mean peak recovery occurred between 80 and 90 minutes following the start of the work loop protocol (Fig 6.4.4).

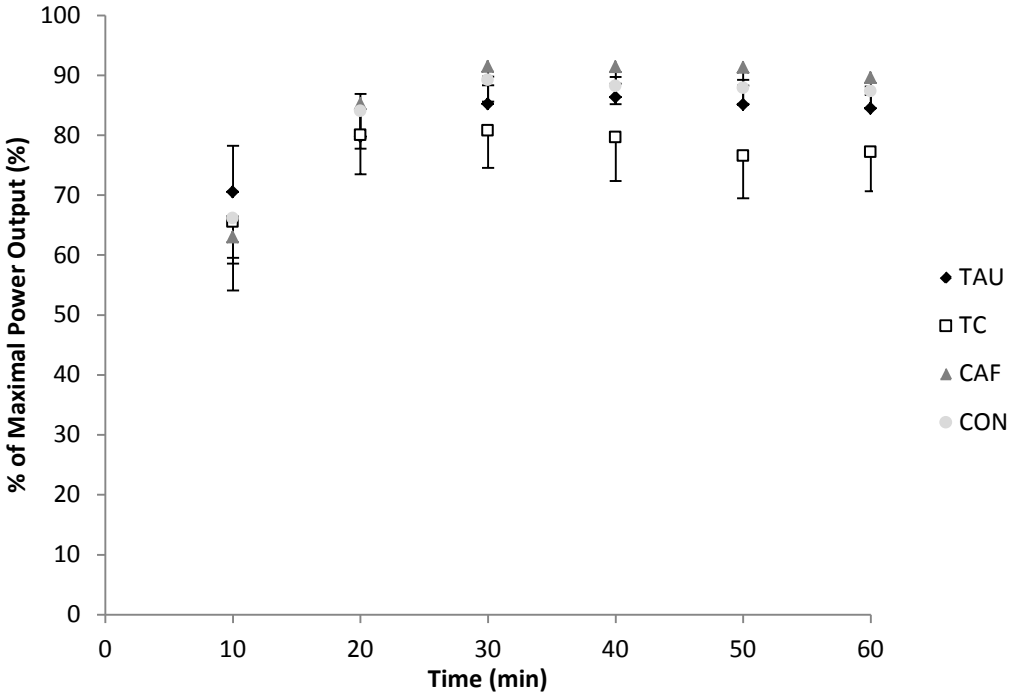


Figure 6.4.4 - Effect of physiological concentrations of taurine alone (TAU), caffeine alone (CAF), and taurine and caffeine combined (TC) on the recovery of mouse soleus muscle following fatigue at maximal stimulation frequency. [Data represented as mean & SE] n = 8 in each case.

6.5. Discussion

The mean maximal tetanus stress of 219 kN $m^{-2}$and net power output of 32.8 ± 1 W kg^{-1}were comparable to those reported in previous studies using similar methods (Askew *et al.* 1997; James *et al.* 2004; Vassilakos *et al.* 2009). More significantly the present results for maximal stress and net power output were directly comparable to 189 and 202 kN $m^{-2}$and 31.7 and33.1 W kg^{-1} previously reported by Tallis *et al.* (2012; 2013; chapter 4 & 5). This signifies that the muscle preparations used across the studies were of similar quality and vindicates the use of the caffeine only results from Tallis *et al.* (2012; 2013; chapter 4 & 5) for comparison to the results reported in the present study from which the caffeine only treatment results are drawn.

The Effect of Physiological Concentrations of Taurine and Caffeine on Muscle Power Output

Treatment of mouse soleus muscle with 2.64mM of taurine failed to elicit any significant increases in mean maximal net muscle power output compared to controls. When taurine was combined with 70µM caffeine, mean maximal net power output was significantly increased by up to 4.1% compared to controls. This acute increase in maximal net power output was not significantly different from the up to 6.4% induced with caffeine treatment alone. Therefore, acute supplementation of taurine did not directly improve short-term maximal muscle net power output and failed to further potentiate the effects of caffeine alone.

The evidence in the present study contradicts the findings of Galler and Hutzler (1990) who demonstrated that physiological intramuscular concentrations of taurine (5mM) increased the submaximal isometric force of pig heart muscle and slow abdominal extensor muscle of crayfish. Similarly to Galler and Hutzler (1990), force-potentiating effects were reported by Bakker and Berg (2002) in skinned rat EDL (relatively fast muscle). In this instance the mechanism of action was attributed to short-term modification of ion channel function and Ca^{2+} homeostasis that has further been confirmed by Camerino *et al.* (2004).Subsequently, Bakker and Berg (2002) and Cuisinier *et al.* (2000) confirmed high concentrations of

taurine (20 mM) acted as a modulator of contractile function by sufficiently increasing the myofilament sensitivity to Ca^{2+}. Additionally, Cuisinier *et al.* (2000) demonstrated that taurine did not modify Ca^{2+} sarcoplasmic reticulum uptake. However, it has been suggested that the antioxidant properties of taurine may reduce oxidative stress on the sarcoplasmic reticulum Ca^{2+} pump (Schaffer, 2010). The concentrations of taurine in this research are markedly higher (5-20mM) than the concentration used in the present study and implementation of dynamic muscle function (i.e work loop power output) may further explain differences between previous research and the present results. As no detectable increase in muscle power output occurred, our evidence suggests that physiological concentrations of taurine at the level used in the present study do not significantly affect skeletal muscle Ca^{2+} handling.

The present results are also contradictory to the findings of Steele *et al.* (1990) who suggested that taurine could further potentiate the effects of caffeine. Steele *et al.* (1990) demonstrated that taurine concentrations greater than 1mM contributed to larger caffeine induced contracture of skinned rat heart tissue. However, it is widely established that the effects of taurine are more profound in heart tissue (Scaffer, 2010). The caffeine concentration used by Steele *et al.* (1990) (10mM) is markedly higher than that used on skeletal muscle in the present study and would be toxic for human consumption (Fredholm *et al.* 1999).

The effect of 70μM caffeine to directly increase skeletal muscle power output has been attributed to a greater release of Ca^{2+} into the intracellular space, an increase in myofibrillar Ca^{2+} sensitivity, a decrease in the sensitivity of the SR Ca^{2+} pump, and an increased SR Ca^{2+} permeability (Tallis *et al.* 2012, 2013; chapter 4 & 5; Allen *et al.* 1989, 1995). We believed by further increasing muscle Ca^{2+} sensitivity via treatment with taurine a greater caffeine induced potentiation would occur. As this wasn't the case this adds further weight to the previous argument that taurine at this concentration is unable to modify soleus muscle Ca^{2+} response.

Treatment with 2.64mM of taurine failed to significantly affect the time to fatigue in maximally stimulated mouse soleus muscle compared to controls. When taurine was combined with caffeine time to fatigue was significantly decreased, although treatment of soleus muscle with caffeine alone resulted in significantly faster time to fatigue than all other treatments. These results suggest that in this instance adding taurine to caffeine may in part block the effects of caffeine alone. Futhermore there was little variation in the total amount of work produced during the fatigue protocol between controls, taurine and taurine and caffeine combined treated muscles. Interestingly this may indicate an ability of taurine to reduce the increased relaxation time previously demonstrated in caffeine treatment alone in chapter 5. Contradictory to the findings in chapter 5, caffeine treated muscles had reduced cumulative work compared to controls. Although this maximally stimulated fatigue of mouse soleus muscle was not significantly different (TTest p=0.14) between the muscles preparations used in chapters 5 and 6, time to fatigue for soleus muscle in the present study was 1 second greater therefore allowing power to be applied over a greater duration and potentially a reason for increased cumulative work. This further highlights in inter-individual variation in fatigue between muscle preparations.

A reduction in skeletal muscle taurine content following prolonged muscle stimulation has previously been established *in vitro* (Kim *et al.* 1986; Matsuzaki *et al.* 2002). Chronic supplementation has been shown to significantly increase muscle taurine concentration resulting in increased muscle force production and partially counteracting the effect of intramuscular taurine depletion that occurs during fatigue (Goodman *et al.* 2009). Yatable *et al.* (2002) used a whole animal model to assess the effects of 7 days of 0.5g/kg taurine supplementation on rat skeletal muscle taurine content. Skeletal muscles of the leg displayed a significant increase in taurine concentration post treatment which was followed by an increased time to fatigue of subsequent treadmill running until exhaustion. Post exercise the supplemented rats demonstrated a reduction in the decrease in skeletal muscle taurine concentration compared to controls. The effect demonstrated from prolonged dietary supplementation of taurine did

not prevail in the present acute study suggesting that any increase in soleus muscle taurine concentration from the given treatment was not sufficient enough to substantially increase total muscle taurine concentration.

Tallis *et al.* (2013; chapter 5) demonstrated a decreased time to fatigue in caffeine treated soleus muscle which was attributed to an increase in eccentric work during the lengthening phase of the work loop. In fatigue under control conditions muscle relaxation time is prolonged due to an increase in intracellular Ca^{2+} concentration (Askew *et al.* 1997). It is believed that this effect is amplified in the presence of caffeine resulting in the muscle being active to a greater extent at the end of shortening, hence greater work is required to stretch the muscle back to its resting length (Tallis *et al.* 2013; chapter 5). This provides a partial explanation as to why TC fatigues faster than controls. A combination of increased eccentric work during fatigue in caffeine treated soleus muscles and a higher starting power output may result in a greater oxidative stress and damage to muscle tissue. Silva *et al.* (2011) demonstrated that chronic supplementation of taurine in rats (1-ml 300mgkg^{-1} per body weight) had a cytoprotective role in exercise induced muscle injury. Although this mechanism is not ruled out by the authors it is unlikely that the taurine concentration used in the present study will increase intramuscular taurine levels enough to generate this mechanism. Moreover, Tallis *et al.* (2012; chapter 4) highlighted the diverse individual acute response to 70µM caffeine and a clear division of responder and non-responders to the treatment. The difference in time to fatigue between CAF and TC are most likely to be due to individual differences in response to the caffeine treatment and the ratio of responders and non-responders between the treatment groups. This is partly supported in the present study by the increase in SE for the TC trial compared to CAF that (Fig 6.4.2).

The Effect of Physiological Concentrations of Taurine and Caffeine on Recovery from Fatigue

Treatment of mouse soleus muscle with taurine (T), caffeine (CAF), or taurine and caffeine (TC) combined failed to significantly increase recovery compared to controls. This is similar to the findings of James *et al.* (2004) who also reported that 70µM caffeine treatment failed to significantly increase the recovery of

both mouse soleus and EDL following fatigue. The established ergogenic benefit of caffeine on direct muscle power output of non-fatigued muscle is not great enough to offset the problems in calcium handling that occur during fatigue (Tallis *et al.* 2012; chapter 4; Allen *et al.* 2008).

Intramuscular taurine concentration will decrease during fatigue (Kim *et al.* 1986; Matsuzaki *et al.* 2002). In light of the present results, it is likely that the given concentration of taurine used in the present study is not great enough to significantly increase intramuscular taurine concentration to a level which would evoke such effects; therefore, there was no enhancement of muscle recovery. With no significant effect of either taurine or caffeine alone, it is unsurprising that combining these treatments also failed to elicit an enhanced fatigue recovery.

Practical Implications of the Present Findings

The present findings infer that taurine does not provide any acute ergogenic effect directly on skeletal muscle. This questions the inclusion of taurine into energy drinks that are designed to promote short-term increases in physical performance. However, further research investigating the effect of taurine on other physiological systems, particularly central effects, should also be considered. Although evidence for the prolonged supplementation of taurine has been well established, achieving this through the regular consumption of caffeine containing energy drinks would not be ideal. Regular consumption of caffeine has been shown to result in habituation and a dampening of the ergogenic effects, therefore increasing muscular taurine via this method may sacrifice the benefit of caffeine (Bell & Mcleellan, 2002). In addition to the high quantity of carbohydrate in such drinks, the present findings and the previous work by Tallis *et al.* (2012, 2013; chapters 4 & 5) have demonstrated that caffeine is likely the key ingredient in energy drinks to provide an acute effect directly on skeletal muscle performance.

Acute treatment of taurine on isolated skeletal muscle failed to elicit any significant effect on mouse soleus muscle performance. One off maximal muscle power output, time to fatigue and recovery from fatigue were all unchanged. Taurine treatment also failed to further potentiate the effects of caffeine on

the same parameters. In conclusion, a physiological level of taurine did not provide any direct short-term ergogenic benefit in isolated skeletal muscle.

7. Is the Age Related Decline in Skeletal Muscle Function Muscle Specific? The Effect of Ageing on isolated Locomotory (EDL) and Respiratory (diaphragm) Skeletal Muscle Performance

7.1. Abstract

The present study is the first to use the work loop technique to examine the alteration in skeletal muscle mechanical properties over a range of ages. The decline in skeletal muscle function occurs relatively early in life and should not be soley considered a syndrome of old age. The present research considers developmental alterations that occur in skeletal muscle function as well as changes at various stages beyond physiological maturity. Measurements of maximal isometric stress, activation and relaxation time, maximal power output, fatigue resistance and recovery are compared in muscle isolated from mice 3, 10, 30 & 50 weeks old. This study uniquely compares the ageing response between peripheral locomotory EDL and core diaphragm muscles to examine if any differences in the ageing effect are related to anatomical location and function. A progressive age related reduction in maximal isometric stress that was of greater magnitude to the decrease in maximal muscle power output occurred in both muscles. Maximal force and power developed earlier in diaphragm muscle compared to EDL, but demonstrated a greater age related decline. These results are also the first to provide evidence to rationalise the equivocal literature that examines the relationship between increasing age and endurance capacity. The present study indicates that skeletal muscle fatigue resistance is age and muscle dependant. Despite this, both EDL and diaphragm displayed a typical trend of increased fatigue resistance from 10 to 30 week old, followed by a decrease thereafter to 50 week old. The recovery of muscle power output post fatigue was significantly greater in 3 week old mice; there was no age dependant effect on recovery of diaphragm muscle. In EDL, the age related decline in muscle performance is prevalent without significant atrophy indicating, at least in part, that ageing affects skeletal muscle contractile performance independently of atrophy.

Key Words: Ageing, Fatigue, Power, Sarcopenia, Work Loop

7.2. Introduction

Sarcopenia is commonly associated with old age and refers to the age related reduction in skeletal muscle function; primarily a loss of muscle mass, strength and a slowing of contractile function that greatly reduces mobility and subsequently the quality of life in elderly populations (Williams et al. 2002). However, it has been established that muscle atrophy and associated decline in skeletal muscle performance can occur as early as 25 years of age in humans, which is greatly accelerated in the later stages of life (Lexell et al. 1995). Despite the maintenance of a physically active lifestyle it is impossible to fully offset the age related decline in muscle function and changes in body composition (Klitgaard et al. 1990). Little is known about the rate of decline in muscle performance between peak performance and 'old age'. Subsequently the present study aims to assess the mechanical properties of mammalian skeletal muscle during development and at various stages post physiological maturity in an attempt to assess early signs of an age related decline in muscle performance.

A reduction in skeletal muscle strength (maximal force in a single attempt) and power (the rate at which work is done; the product of force produced and the speed of muscle shortening) are commonly established effects of the ageing process (Doherty, 2003; Deschenes, 2004). Murray et al. (1980; 1985) reported that isometric strength of the knee flexor and extensor muscles reduced by an average of 55-65% and 56-78% in elderly men and women respectively. A range of human and in vitro research studies using mammalian muscle isolations have supported the age related reduction in the maximal force generating capacity (Brooks & Faulkner, 1988; Zhang & Kelsen 1990, Gonzalez & Delbobo, 2001). Some studies have demonstrated that the decline in muscle power is significantly faster than the loss of strength, which has been in part attributed to a reduction in the muscle force-velocity relationship and maximal unloaded shortening velocity (V0) (Metter et al. 1997; Thompson and Brown, 1999; Krivickas, 2001; Raj et al. 2010). However, Brooks and Faulkner (1988) demonstrated a reduction in mouse EDL (by 78%) and soleus (by 73%) muscle power output without changes in the force-velocity relationship.

The evidence investigating the effect of ageing on muscle endurance (the ability of the muscle to resist fatigue) is somewhat equivocal, and discrepancies in results are likely to stem from the variation in experimental methods. Differences are apparent in the fatigue protocol, the duration for which muscle endurance is measured and the muscle groups tested (Deschenes, 2004). Izquierdo et al. (2001) reported a significant reduction (19 % when related to body mass) in the maximal workload of elderly subjects following a multistage incremental cycling test. A similar relationship between increasing age and reduced skeletal muscle endurance has been established in other in vivo research (Lennmarken et al. 1985; Davies et al. 1986). In contrast Lindstrom et al. (1997) failed to demonstrate any significant effect of ageing on muscular endurance of the knee extensor muscles of elderly subjects despite the characteristic reduction in maximal voluntary contraction; findings further supported by Backman et al. (1995) and Bemben et al. (1996). Furthermore in vitro research by Pagala et al. (1998) reported a significant increase in the fatigue resistance of rat soleus muscle stimulated via repeated isometric contractions. Interestingly, this did not correspond to the reduction in whole animal endurance in a running and swimming task. Pagalaet al. (1998) therefore concluded that the age related increase in fatigue was attributed to central or extra-muscular processes. Despite this an increased fatigue resistance following isometric contraction of ankle dorsiflexor muscle in older human subjects has been demonstrated without subsequent reduction in neural activation (Kent-Braun et al. 2002).

As ageing affects a number of physiological systems, the degenerative process resulting in sarcopenia is likely to arise from numerous molecular and cellular factors. Muscle wasting correlates strongly to an age related reduction in muscle performance. It has been suggested that a reduction in motor unit number and size, decreased protein synthesis, disuse atrophy mechanisms, and alterations in hormone production make up a number of interacting factors that result in muscle wasting (Gray et al. 1991; Veldhuis et al. 1997; Welle et al. 1998; Navarro et al. 2001). Furthermore, age related changes in muscle contractile performance also seem to be related to both a shift to a more oxidative muscle composition and a reduction in the excitation contraction coupling performance (Aniansson et al. 1986; Coggan et al. 1991; Navarro et al. 2001; Short et al. 2005).

Although the effects of sarcopenia are well documented within the literature, discrepancies in the evidence exist as to the level at which muscle performance is lost. Much of the previous ageing research that measure muscle contractility in rodents compare a physiologically mature population against an aged population and such use rodents older than those in the present study. The current research aims to uniquely examine skeletal muscle function at various time points between physiological maturity and 'old age' in order to develop a greater understanding of the ageing process and determine the rate of decline in muscle performance beyond physical maturity. Furthermore studies that assess age related changes in muscle power output in isolated muscle are scarce, and calculations of muscle power constructed form force velocity data and maximal isometric force, as by Brooks and Faulkner (1998), have been demonstrated to be poor estimates of power output obtained from the work loop technique (James et al. 1996).The present study is the first to use the work loop technique to form a closer representation of in vivo muscle usage (Josephson, 1985) to improve our understanding of the age related changes in skeletal muscle performance. Although research examining the phenomenon of skeletal muscle ageing using isometric measures has provided a valuable insight, this form of muscle action is relatively rare in vivo and may relate to the discrepancies in relating these results to whole animal performance (Chan & Head, 2010). Furthermore, it is likely that the age related decline in muscle performance is muscle specific, the greater reduction in the cross sectional area of type II fibers suggests that skeletal muscle with a predominantly faster fiber type will show the greatest response to ageing (Aniansson et al. 1986; Alnaqeeb & Goldspink, 1986; Coggan et al. 1991). In addition, beyond physiological maturity increasing age is associated with a reduced physically active lifestyle and as a consequence related disuse atrophy might play an important part in the loss of locomotory muscle performance that may not be as predominant in core respiratory skeletal muscle.

The present study aims to use isolated mouse EDL (relatively fast fiber type) and diaphragm (more mixed fiber type) muscle to build a unique profile of the age related changes in muscle mechanical properties that occur at various age between developmental and 'middle aged' mice by examining changes in: 1) maximal isometric force and dynamic power output; 2) muscle activation and relaxation time; 3) fatigue

of muscle power output; 4) post fatigue recovery. By eliminating CNS, peripheral nerve, and neuromuscular junction innervations the present study will quantify the magnitude of the ageing response by direct stimulation of skeletal muscle.

By making further comparisons between mouse EDL and diaphragm muscle the present study is also the first to assess the effects of increasing age between a peripheral and a core muscle with different anatomical location, function and predominant fiber type. It is predicted that the rate of muscle development and the reduction in performance due to increasing age will be muscle specific as locamotrory muscle may be subject to disuse atrophy mechanisms to a greater degree than core diaphragm muscle. Furthermore difference in predominant fiber type expression between EDL and diaphragm are likely to be subjected to developmental changes at different rates. Increasing age beyond physiological maturity will be associated with a reduction in maximal muscle power output (and corresponding muscle activation and relaxation time) that will be greater in magnitude than the reduction in maximal force. The effect of increasing age on fatigue resistance will again be muscle specific; generally muscle will show decreased endurance ability.

7.3. Materials and Methods

A more detailed account of the methods is given in the general methods section (chapter 3).

From birth mice were housed in groups of 8 without access to running wheels and aged to 3 weeks, 10 weeks, 30 weeks, and 50 weeks (n = 20 for each age group) before sampling. Pups were weaned 21 days postpartum, thus 3-week-old mice were used to represent muscle in a developmental stage. 10 week old mice where used to represent physical maturity. Mice at 50 weeks were used to represent 'middle age'. Results presented by Lang & White (2000) demonstrate a good survival rate of CD1 mice during the first 12 months. At 50 weeks survival rates were above 85%, however beyond this point mortality rate increased more rapidly. Lang & White (2000) further showed a mortality rate of approximately 50% at 18 months, thus mice beyond this would be deemed 'old aged' and are representative of rodents used in previous work (Brooks & Faulkner, 1988; Gonzalez et al. 2000; Gonzalez & Delbono, 2001). 12 month old mice were also used to represent a 'middle aged' group by Gonzalez et al. (2000) who investigated the reduction in specific force of EDL and soleus muscle fibers.

Either EDL muscle (9.3% type IIX, 86.8% type IIB in 90 day old mice; Agbulut et al. 2003) was dissected from the left hind limb and immediately frozen in liquid nitrogen. EDL from the right hind limb was used in the muscle mechanics assessments. Or whole diaphragm muscle (15.6% type I, 43.6% type IIA, 34.6% type IIX, 6.2% type IIB in 90 day old mice; Agbulut et al. 2003) was dissected from a separate mouse and subsequently split in half; the right hand side was immediately frozen in liquid nitrogen while the central portion of the second section was used in the muscle mechanics protocol: aluminium foil T-clips were wrapped around the central tendon at one end, however, at the opposing end two ribs anchoring the muscle were left intact.

Once dissected the muscle was placed in the muscle bath in circulated oxygenated Krebs solution at 37^{o}C. Muscle length and stimulus amplitude (14-18V for EDL; 10-16V for diaphragm; current fixed at 160 mA) were optimised in order to achieve maximal isometric twitch force.

Maximal isometric tetanic force was measured by subjecting each muscle preparation to a 250ms burst of electrical stimulation. The frequency of stimulation was further optimised in order to yield maximal tetanic force; this was usually 200Hz for EDL, 140Hz for diaphragm and did not change with age. Time to half peak tetanus (THPT), or the time from the start of force production until half the peak force is achieved, and time from last stimulus to half tetanus relaxation (LSHR), the time between the end of stimulation and the reduction in force to half of maximum, were measured as indicators of muscle activation and relaxation time. A 5-minute rest period was imposed between each tetanus in order to allow sufficient time for the muscle to recover.

All EDL and diaphragm muscle followed this process of isometric measures before proceeding on to the subsequent work loop protocol.

The Work Loop Technique

A cycle frequency of 10Hz and 7Hz was used for EDL and diaphragm muscle, respectively. 10Hz represents the cycle frequency that has been previously shown to elicit maximal power output in isolated mouse EDL (James et al. 1995). 7Hz was the cycle frequency found to elicit maximal power concurrent with the finding of Altringham and Young (1991). Usually a 49ms burst of electrical stimulation applied through the shortening phase of the work loop was used for EDL in keeping with that used at 10Hz cycle frequency in James et al. (1995). The burst duration commonly used to elicit maximum power output in diaphragm muscle was 55 ms. On occasions the burst duration had to be increased or decreased to adjust the number of stimuli given to maximise power output in individual muscle preparations. A stimulation phase shift of -2 ms and -5 ms were used for EDL and diaphragm respectively as they elicited maximal power output.

The work loop protocol lasted 180 minutes; during this time assessments of each muscles maximum power output, response to 70µM caffeine treatment, fatigability, and recovery from fatigue were made (Fig 7.3.1).

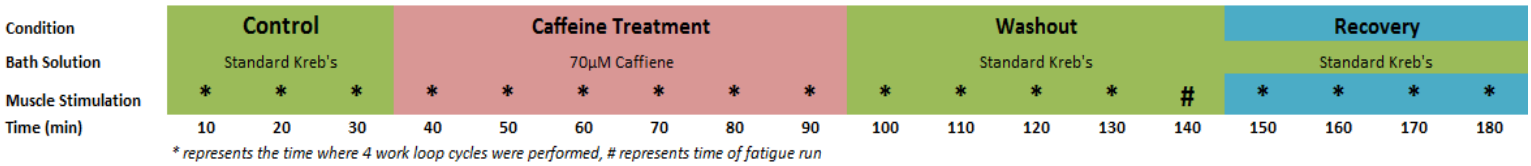


Figure 7.3.1. - Schematic of the work loop protocol to examine fatigue when maximally stimulated, the recovery of maximal muscle power output after fatigue, and the ergogenic effect of 70µM caffeine on acute muscle power output in developmental and middle aged mouse EDL and Diaphragm muscle

Maximal Acute Power: The parameters employed to elicit maximal work loop power output for both EDL and diaphragm have been outlined above. Each muscle was subjected to four sinusoidal length change cycles at 10-minute intervals until maximal muscle power output was achieved. It can take time for muscle to equilibrate to the protocol; however, maximal power output was usually achieved within 3 measurements. The third work loop of each set of four typically produced the highest power and was therefore taken as the indicative measure of muscle power output in all work loop experiments. A 10-minute rest interval was imposed between each set of four work loops in order to allow the muscle sufficient recovery time.

The Effect of 70µM Caffeine Treatment: The protocol (Fig 7.3.1.) to test the effects of 70µM (physiological maximum) different aged muscle was 130 minutes. The methods and results are discussed in a separate chapter (Chapter 8).

Time to Fatigue: Following the treatment of each muscle preparation with caffeine the muscle was allowed to equilibrate in standard Krebs-Henseleit solution for 40 minutes prior to the fatigue run (Fig 7.3.1.). This time duration has previously been shown to adequately washout any effects of 70µM caffeine treatment (James et al. 2005; Tallis et al. 2012; chapter 4). In order to test fatigability each muscle was then subjected to 50 consecutive work loop cycles using the stimulation and length change parameters that elicited maximal power output. In light of our previous findings in chapter 2, 50 consecutive work loops was deemed adequate to induce fatigue in the present experimental muscles. Data was recorded

for every second loop until force had significantly reduced and a plateau occurred, or until net work was negative. In contrast to the methods in chapters 5 and 6 fatigue was plotted until net power was below 50% as not all muscles fatigued until net negative work was produced and it was difficult to accurately determine where the plateau had occurred.

Recovery from Fatigue: The ability of the muscle to recover from fatigue was monitored for 30 minutes following the fatigue run. Three measurements of maximal work loop power output were made every 10-minutes and were compared directly to the pre fatigue maximum muscle power output (Fig 7.3.1).

At the end of the experiment the muscle was detached from the rig, tendons removed, then weighed in order to calculate isometric stress (kN.m-2) and normalised muscle power (W.kg-1).

Statistical Analysis of Data

In order to assess the effect of age on isometric twitch stress, maximal tetanus stress, isometric times, and maximum work loop power output single factor ANOVA were conducted in SPSS (Version 16, SPSS inc., IL, USA) on each data set for EDL and diaphragm. Tukey post hoc tests were conducted to see where specific differences occurred. Unpaired TTests were used at each age to identify differences in these parameters between EDL and diaphragm muscles.

Separate two factor ANOVA were conducted on each muscle to determine if there was an effect of age on time to fatigue/recovery from fatigue, and if muscle power output changed overtime. Tukey post hoc tests were conducted on age in order to identify where these differences occurred. In the present study muscle fatigue was defined as when the muscle produced less than 50% of its pre fatigued maximal power output. Therefore, statistical analysis was only performed on the data up to this point.

Results were interpreted as significant when $p < 0.05$. Values are displayed as mean ± standard error.

7.4. Results

Body & Muscle Mass

Increasing age resulted in a significant increase in mean mouse body mass (Fig 7.4.1.). At 50 weeks of age mean body mass had increased by 73% compared to that at 3 weeks of age. The largest increase occurred between 3 and 10 weeks of age with the mean body mass of 10-week-old mice being 50% greater than 3-week-old mice (Fig 7.4.1; Tukey $p<0.001$). A statistical tendency for an increase in body mass (by 23%) was shown between 10 and 30 weeks of age (Fig 7.4.1; Tukey $p=0.076$). Mean body mass at 50 weeks of age was significantly higher (by 28%) compared to that at 30 weeks of age (Fig 7.4.1; Tukey $p<0.001$). By 50 weeks of age individual body masses had either increased above 70g (Fig 7.4.1; A) or appeared to stay below 50g (Fig 7.4.1; B) which is similar to the mean body mass at 30 weeks of age. If the 7 individuals in-group A are omitted and mean body mass between 30 weeks of age and 50 weeks of age is compared, there is no significant difference (Fig 7.4.1; TTest $p=0.89$). These two distinct groups at 50 weeks infer that body mass will either plateau or continue to significantly increase.

For EDL, muscle mass was significantly affected by age (ANOVA$p<0.001$). Mean muscle mass at 3 weeks of age was 6.7±0.5mg and significantly lower than all other age groups (Tukey $p<0.001$ in all cases). Peak muscle mass was 16±0.8mg and occurred in animals aged 50 weeks of age, this proved to be significantly greater than the 12.4±0.4mg at 10 weeks of age. Similar measures were not made for diaphragm as dissection affected the size of the muscle preparation and part of each diaphragm was fast frozen.

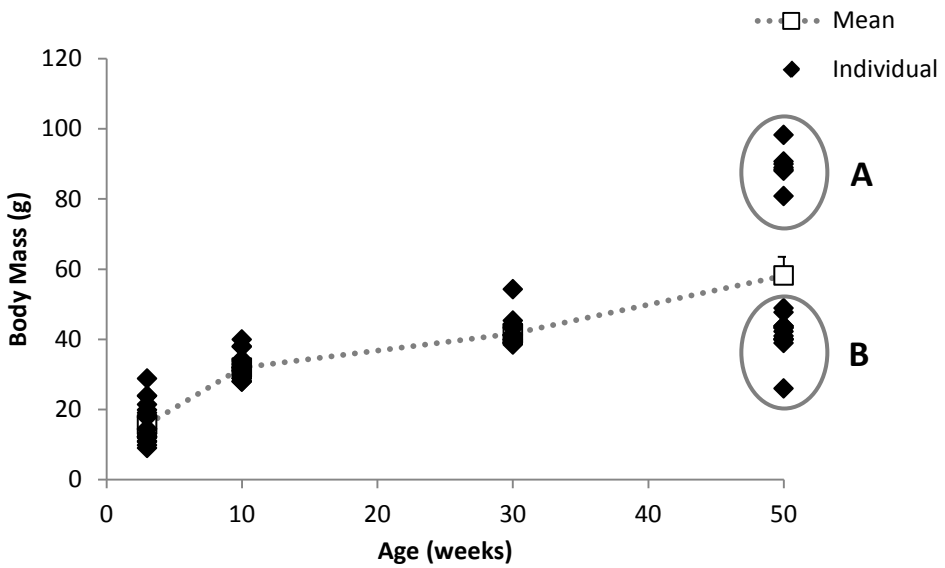


Figure 7.4.1 – The effect of age on the mean and individual body mass of CD1 mice. A subdivision in the 50 week data is highlighted by A & B. Data labelled A represents 50 week old mice with body mass greater than 70g, whereas data labelled B represents 50 week old mice with body mass below 50g [Data represented as mean ± SE; n=20 for each age group]

Maximal Isometric Twitch Stress

Mean twitch stress was significantly affected by age in both EDL and diaphragm muscle (Fig 7.4.2; ANOVA $p<0.006$ in both cases). For EDL mean twitch stress was greatest in mice at 10 weeks of age and was significantly reduced (by 39%) at 3 and (by 27%) 50 weeks of age (Fig 7.4.2; Tukey $p<0.007$ in both cases). Furthermore EDL twitch stress had a tendency to be reduced (by 20%) at 30 weeks of age when compared to 10 weeks of age (Fig 7.4.2; Tukey $p=0.054$).EDL stress in 3-week-old mice had a tendency to be lower than at 30 weeks of age (Fig 7.4.2; Tukey $p=0.063$), but was not significantly different to 50 weeks of age (Fig 7.4.2; Tukey $p=0.336$). There was no significant difference between twitch stresses at 30 and 50 weeks of age (Fig 7.4.2; Tukey $p=0.814$).

In diaphragm muscle mean twitch stress was greatest at 10 weeks of age and had significantly reduced at 30 (by 34%) and 50 weeks of age (by 27%) (Fig 7.4.2; Tukey $p<0.04$ in both cases). There were no further significant differences in mean twitch stress between 3, 10, 30 and 50 weeks of age (Fig 7.4.2 Tukey $p>0.17$ in all cases).

Mean isometric twitch stress of EDL was significantly higher than diaphragm at 10, 30 and 50 weeks of age (Fig 7.4.2; TTest $p<0.001$ in each case).However, at 3 weeks of age there was no significant difference in mean isometric twitch stress between EDL and diaphragm (Fig 7.4.2; TTest $p=0.15$).

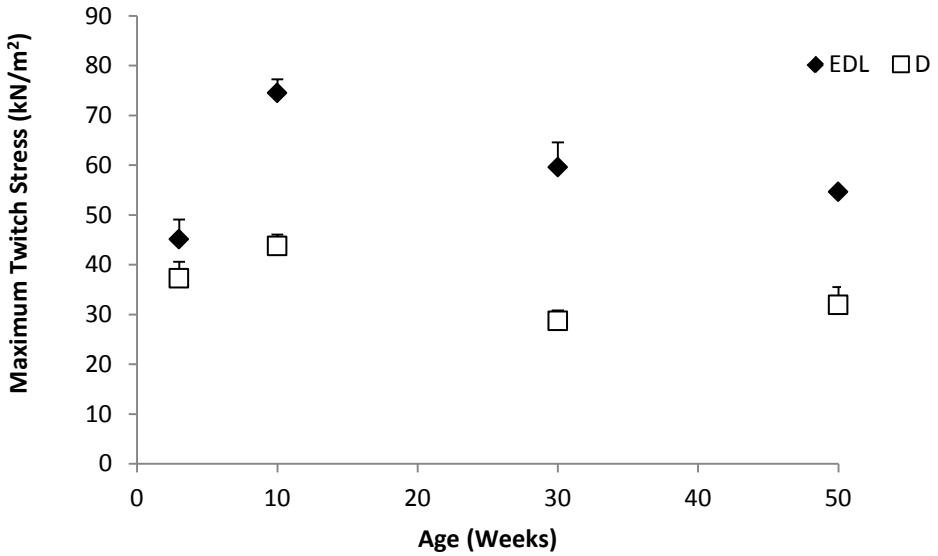


Figure 7.4.2 – The effect of age on mean maximal isometric twitch stress of mouse EDL and diaphragm muscle [Data represented as mean ± SE: n=10 in each case

Maximal Isometric Tetanus Stress

For both EDL and diaphragm mean maximal isometric tetanus stress was significantly affected by age (Fig 7.4.3; ANOVA $p<0.03$ in each case). For EDL mean maximal isometric tetanus stress was achieved at 10 weeks of age, but had significantly reduced by 50 weeks of age (by 22%) (Fig 7.4.3; Tukey $p=0.025$) and

had a tendency to be lower at 3 (by 17%) and 30 weeks of age (by 18%)(Fig 7.4.3; Tukey $p<0.1$ in both cases). There were no significant differences in maximal isometric tetanus stress between 3, 30 and 50 weeks of age (Fig 7.4.3; Tukey $p>0.9$ in all cases). At 10 weeks maximal isometric twitch stress was significantly higher than the value at 8 weeks reported in chapter 4 (Tukey $p<0.005$).

In the diaphragm muscle the highest value for mean maximal tetanus stress was achieved at 10 weeks of age andwas significantly reduced at 30 (by 28%) and 50 weeks of age (by 33%) (Fig 7.4.3; Tukey $p<0.004$ in both cases).There was no significant difference in mean tetanus stress between 3 weeks of age and 10, 30 weeks of age (Fig 7.4.3; Tukey $p>0.1$ in both cases). However, mean tetanus stress at 3 weeks of age was significantly greater than at 50 weeks of age (Fig 7.4.3; ANOVA Tukey $p=0.024$). Furthermore there was no significant difference in stress between 30 weeks of age and 50 weeks of age (Fig 7.4.3; Tukey $p=0.924$).

When compared to diaphragm, EDL produced significantly greater isometric tetanus stress at each age group (Fig 7.4.3 TTest $p<0.001$ in all cases).

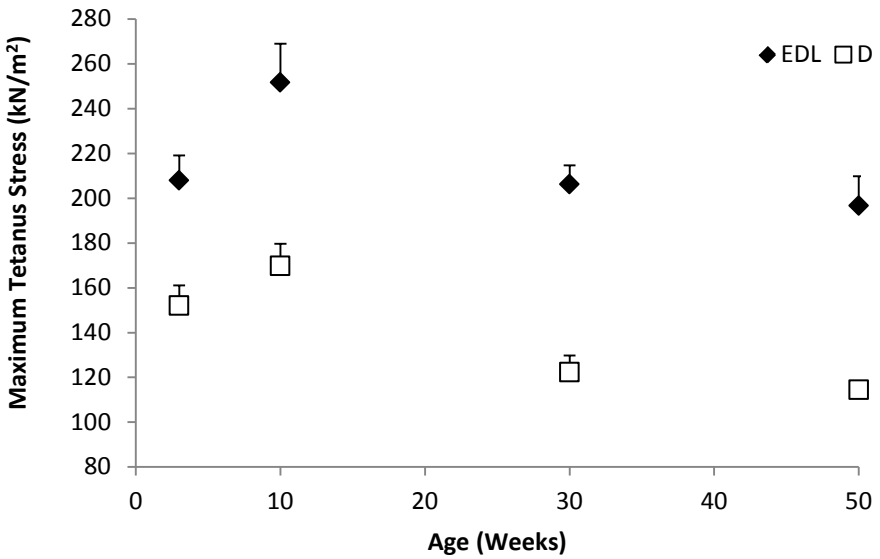


Figure 7.4.3 – The effect of age on mean maximal isometric tetanus stress in mouse EDL and diaphragm [Data represented as mean ± SE: n=10 in each case)

Isometric Activation and Relaxation Times

For EDL mean Time to Half Peak Tetanus (THPT) and Last stimulus to Half Relaxation (LSHR) were significantly affected by age (Fig 7.4.4; ANOVA $p<0.004$ in both cases). Mean THPT at 3 weeks of age was significantly longer (by up to 46%) than at 10, 30, and 50 weeks of age (Fig 7.4.4A; Tukey $p<0.001$ in all cases). THPT was not significantly different between 10, 30 and 50 weeks of age (Fig 7.4.4A; Tukey $p>0.6$ in each case). LSHR was significantly prolonged at 50 weeks of age(by up to 32%) compared to 3, 10 and 30 weeks of age (Fig 7.4.4B; Tukey $p<0.05$ in all cases). There were no further significant differences in THPT between 3, 10 and 30 weeks of age (Fig 7.4.4; Tukey $p>0.6$ in all cases).

In diaphragm muscle mean THPT was significantly affected by age (Fig 7.4.4; ANOVA $p=0.036$). Mean THPT was significantly longer (by 19%) at 30 weeks of age compared to at 10 weeks of age (Fig 7.4.4; Tukey $p=0.021$). There were no further significant differences in THPT between 3, 10, 30, and 50 weeks of age (Fig 7.4.4; Tukey $p >0.3$ in each case). For diaphragm muscle mean LSHR was not significantly different between age groups (ANOVA $p = 0.103$).

Mean THPT and LSHR times were significantly shorter in EDL compared to diaphragm at each age tested (TTest $p<0.001$).

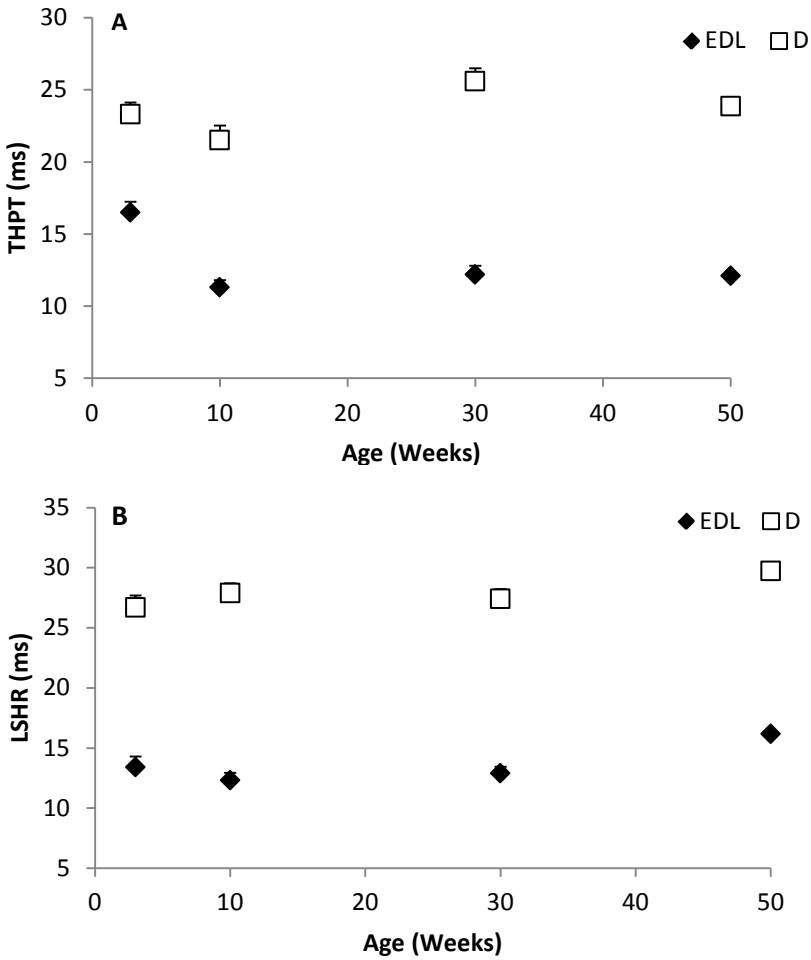


Figure 7.4.4 – The effect of age on mean isometric tetanus muscle activation time (A: THPT; time to half peak tetanus) and relaxation time (B: LSHR; last stimulus to half tetanus relaxation) in mouse EDL and diaphragm muscle [Data represented as mean ± SE: n=10 in each case)

Work loop Power Output

Work Loop Power Output Normalised to Muscle Mass (Watts/kg)

For both EDL and diaphragm mean maximal work loop power output, normalised to muscle mass, was significantly affected by age (Fig 7.4.5; ANOVA $p<0.025$ in both cases). For EDL mean maximal work loop power output peaked at 10 weeks of and was significantly higher than at 3 weeks of age (Fig 7.4.5; Tukey $p=0.014$).Despite a 13% decrease in mean work loop power output by 50 weeks of age this did not prove to be significantly different to power output at 10 weeks of age (Fig 7.4.5; Tukey $p=0.162$).

In diaphragm mean maximal work loop power output was achieved at 10 weeks of age and was significantly reduced at 50 weeks of age (by 23%) (Fig 7.4.5; Tukey p=0.012). Work loop power output at 3 weeks of age had a tendency to be greater than that 30 weeks of age (Fig 7.4.5; Tukey p=0.064), otherwise there were no further statistical significances in this data set (Fig 7.4.5; Tukey p>0.4 in all cases).

Maximal work loop power was greater at 10 weeks compared to 8 weeks as presented in chapter 4 (Tukey p=0.045). Furthermore, mean work loop power output was significantly greater in EDL compared to diaphragm at 3, 10, 30 and 50 weeks of age (Fig 7.4.5; TTest p<0.001 in all cases).

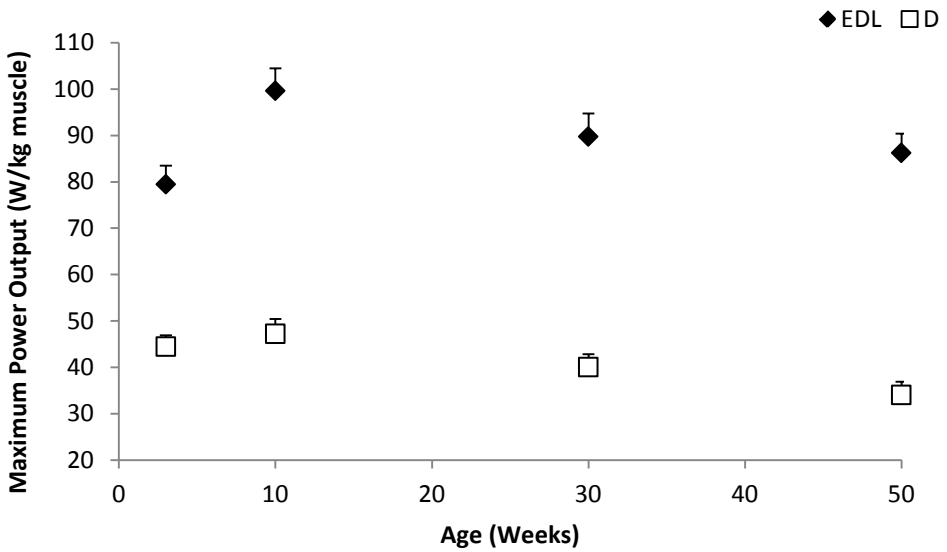


Figure 7.4.5 – The effect of age on mean maximal work loop power output plotted as Watts per kilogram muscle mass for mouse EDL and diaphragm muscles [Data represented as mean ± SE: n=10 in each case)

Work Loop Power Output Normalised to Whole Animal Body Mass (Watts/g)

Locomotory muscles produce power to move the mass of the body, therefore, a key issue is the ability of the muscle to produce power relative to the mass of the body. Mean muscle PO, normalised to body mass, was significantly affected by age for EDL muscle (Fig 7.4.6; ANOVA p<0.026). For EDL mean maximal

work loop power output, when normalised to body mass, was highest at 10 weeks of age and had significantly decreased (by 22%) at 50 weeks of age (Fig 7.4.6; Tukey p=0.023). Furthermore body mass specific power output at 3 (by 20%) and 30 weeks (by 19%) of age had a tendency to be reduced (Fig 7.4.6; Tukey p<0.07) in comparison to 10 weeks of age. There was no significant difference in mean body mass specific muscle power output of EDL between 3, 30 and 50 weeks of age (Tukey p>0.9 in all cases).

Similar calculations cannot be made for diaphragm muscle as whole diaphragm muscle mass was not measured.

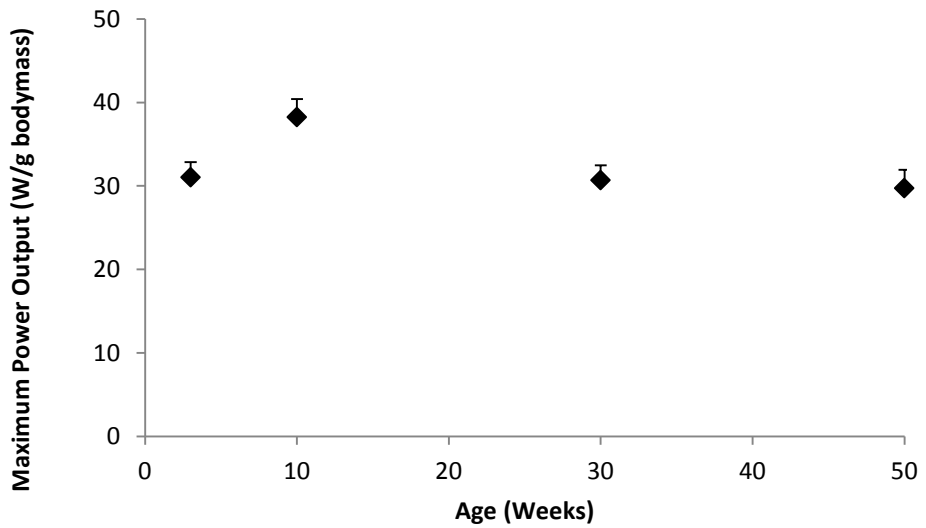


Figure 7.4.6 – The effect of age on mean maximal work loop power output plotted as Watts per gram body mass for mouse EDL [Data represented as mean ± SE: n=10)

The Effect of Increasing Age on Work Loop Shape of Maximally Stimulated Muscle

The area of the work loop represents the net work of that particular cycle; the typical work loop shapes presented in Figure 7.4.7 clearly demonstrate that for EDL net work is the greatest at 10 and 30 weeks, net work is reduced at 50 weeks and is again lower at 3 weeks (Figure 7.4.7; A). Similarly for diaphragm (Figure 7.4.7; B) net work is greatest at 10 weeks and this is similar to that at 30 weeks. Net work is considerably reduced at 3 and 50 weeks. The shapes of the loops indicate that 30 and 50 week EDL

(Figure 7.4.7; A) show an increased relaxation time, a phenomenon that is not as pronounced in diaphragm muscle (Figure 7.4.7; B).

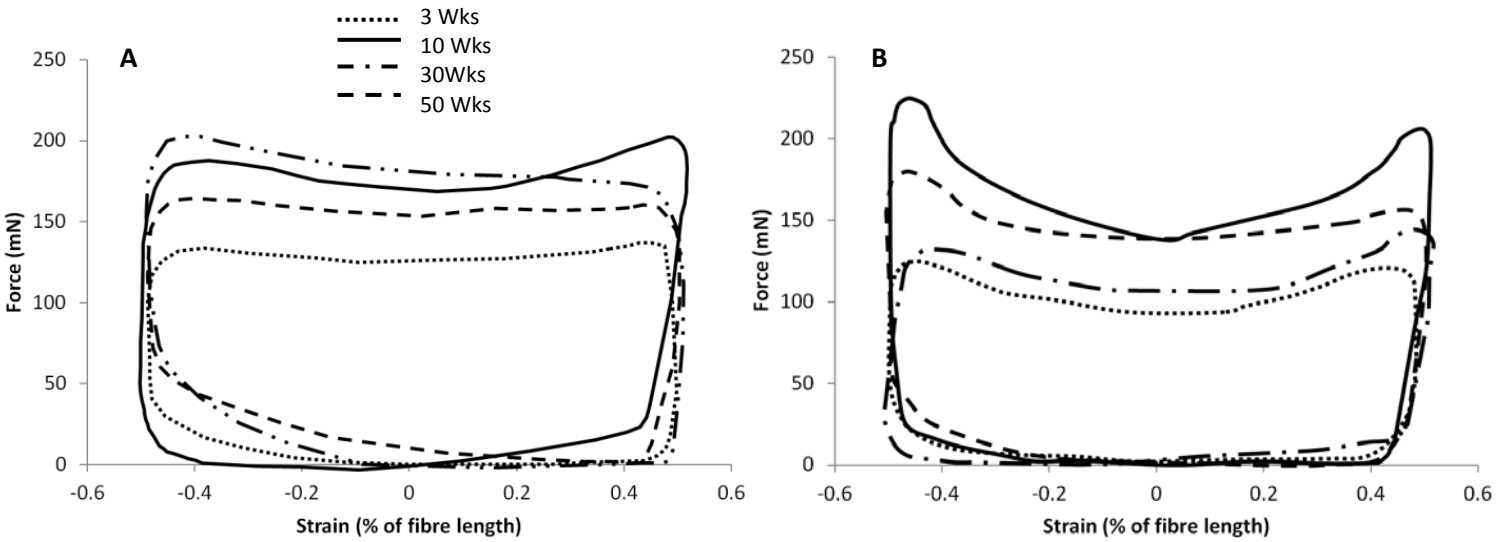


Figure 7.4.7 - The effect of age on typical work loop shapes of: A) mouse diaphragm maximally stimulated at 7Hz cycle frequency and B) EDL maximally stimulated at 10Hz cycle frequency

Fatigue Resistance

The fatigue protocol resulted in a significant reduction in mean muscle power output over time for both EDL and diaphragm muscle (Fig 7.4.8 & 7.4.9; ANOVA $p<0.001$ in both cases). Time to fatigue was also significantly affected by age in both EDL and diaphragm muscle (Fig 7.4.8 & 7.4.9; ANOVA $p<0.001$ in both cases).

For EDL, time to 50% fatigue at 50 weeks of age was significantly faster than that at 3 (by 31%), 10 (by 18%) and 30 weeks of age (by 25%)(Fig 7.4.8; Tukey $p<0.001$ in all cases). Time to 50% fatigue of EDL at 10 weeks of age was significantly faster than 3 (by 15%) and 30 weeks of age (by 8%)(Fig 7.4.8; Tukey $p<0.001$ in each cases). There was no significant difference in time to 50% fatigue between 3 weeks of age and 30 weeks of age (Fig 7.4.8; Tukey $p=0.97$).

In diaphragm muscle time to 50% fatigue was significantly faster at 10 weeks of age compared to 3 (by 27%), 30 (by 15%) and 50 (by 9%) weeks of age (Fig 7.4.9; Tukey $p<0.001$ in all cases). Mean time to

fatigue at 50 weeks of age was significantly faster than at 3 (by 21%) and 30 weeks of age (by 8%) (Fig 7.4.9; Tukey $p<0.001$ in both cases). Diaphragm at 30 weeks of age fatigued significantly faster than that at 3 weeks of age (Fig 7.4.9; Tukey $p=0.001$).

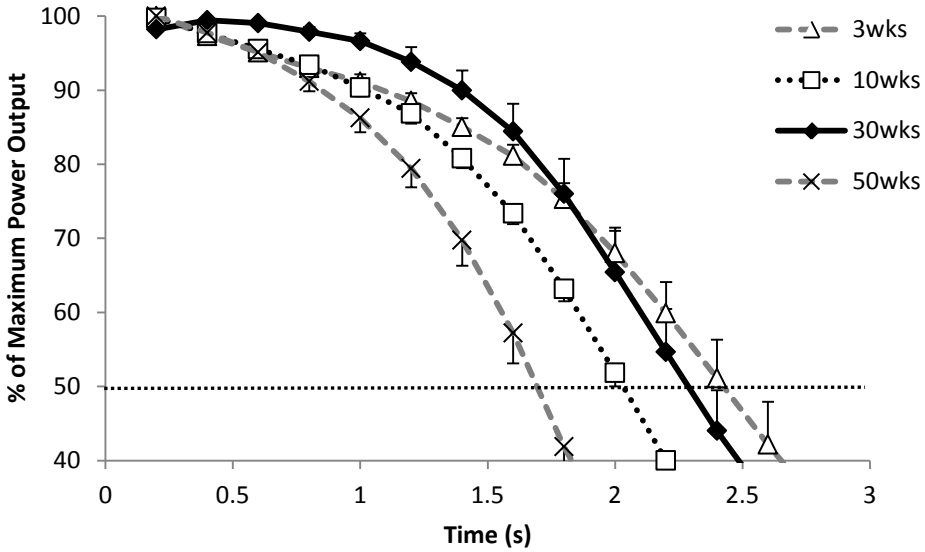


Figure 7.4.8 – The effect of age on fatigue of maximal muscle power output in mouse EDL [Data represented as mean ± SE: n=10 in each case; wks = weeks of age)

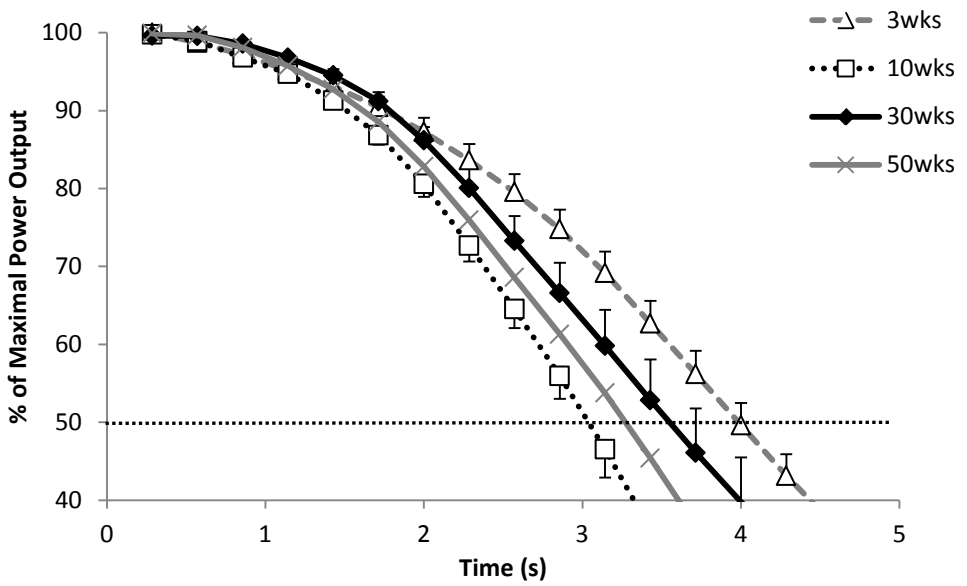


Figure 7.4.9 – The effect of age fatigue of maximal muscle power output in mouse diaphragm [Data represented as mean ± SE: n=10 in each case; wks = weeks of age

Figures 7.4.10 & 7.4.11 depict typical work loop shapes at various points of the fatigue run for EDL and diaphragm muscle, whereby in general fatigue is associated with reduced net work over time as the area of the work loops reduce. 3 week EDL (Figure 7.4.10; A) had the smallest reduction in the area of the work loop with fatigue, this is also associated with minimal change in muscle relaxation time over this period. Work loop shapes from 10 and 50 weeks (Figure 7.4.10; B & D) demonstrate a much larger increase in muscle relaxation time over the time course of the fatigue run. The area of the work loop 18 appears to be less at 50 weeks (Figure 7.4.10; D) compared to other age groups. For diaphragm muscle the work loop shapes indicate that fatigue is associated with an increased relaxation time, which is more greatly pronounced in 10 week old muscle (Figure 7.4.11; B). Changes in relaxation time during fatigue at 30 weeks appear to be minimal (Figure 7.4.11; C).

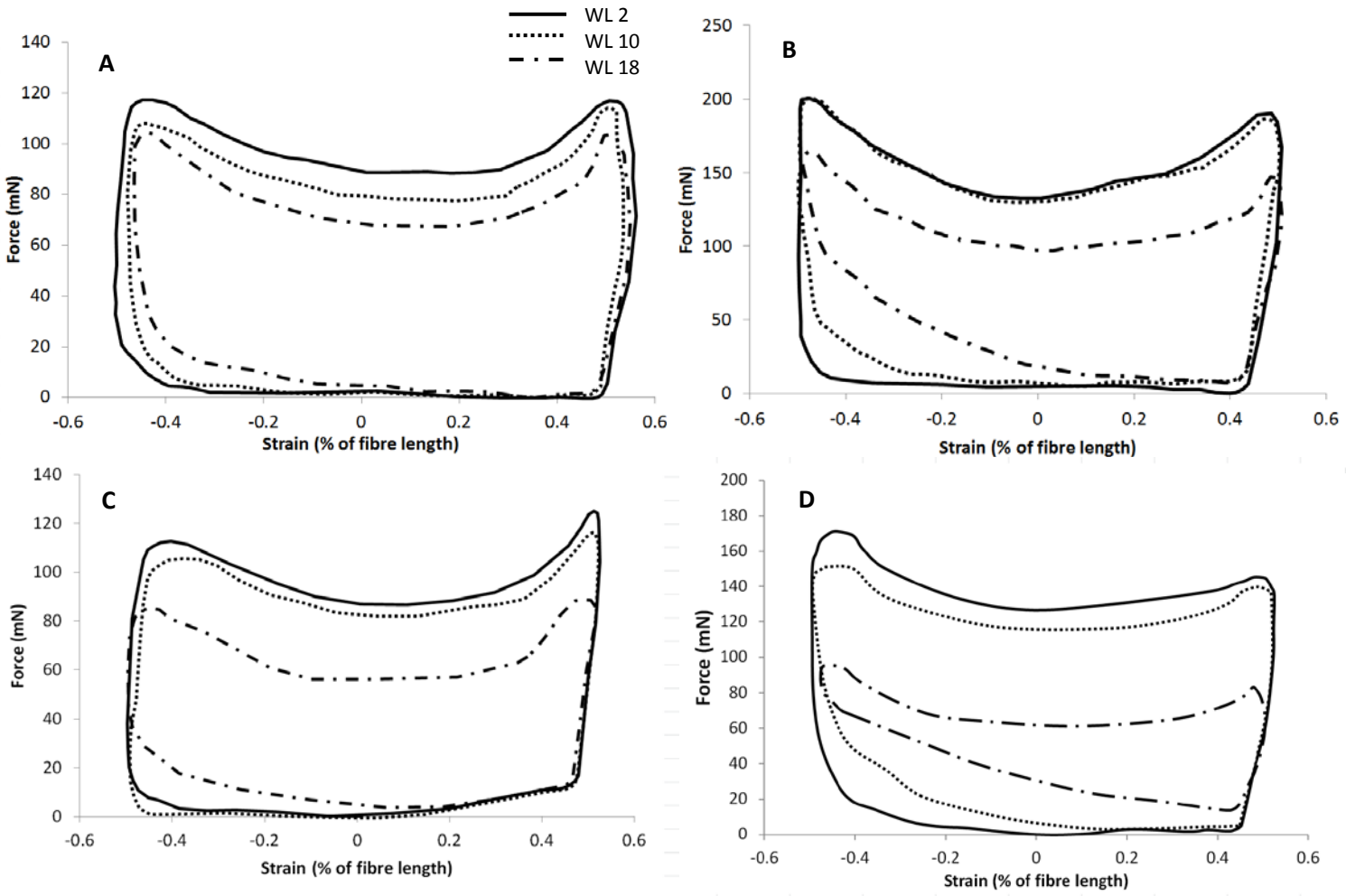


Figure 7.4.10 - The effect of age on typical work loop shapes of mouse EDL muscle maximally fatigued at 10Hz cycle frequency; (A) 3 week old mice (B) 10 week old mice (C) 30 week old mice (D) 50 week old mice; the figures depict work loops 2 (0.2s of the fatigue run), 10 (1s) and 18 (1.8s) of the fatigue run

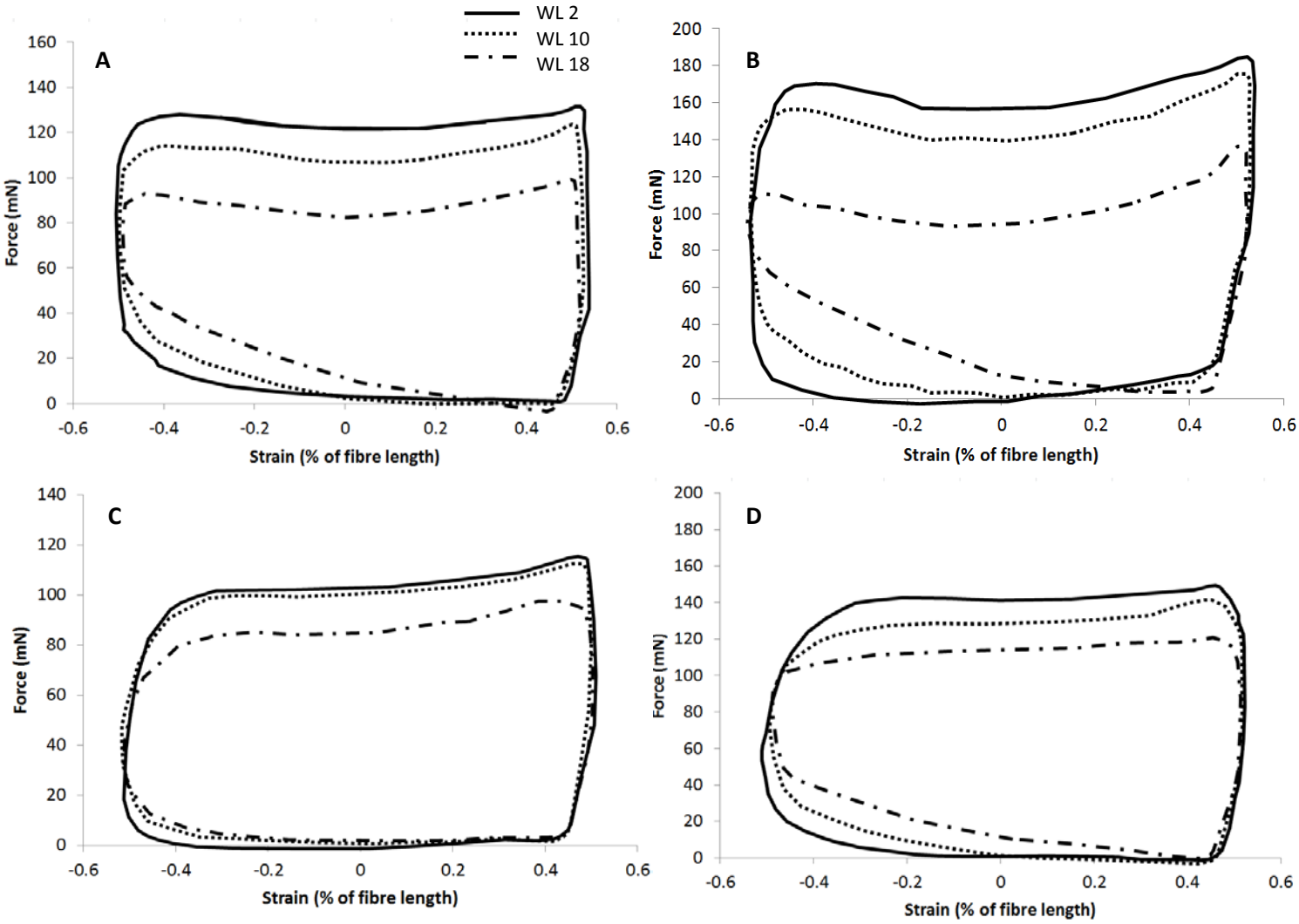


Figure 7.4.11 - The effect of age on typical work loop shapes of mouse diaphragm muscle maximally fatigued at 7Hz cycle frequency; (A) 3 week old mice (B) 10 week old mice (C) 30 week old mice (D) 50 week old mice; the figures depict work loops 2 (0.29s of the fatigue run), 10 (1.43s) and 18 (2.57s) of the fatigue run

Although EDL muscle from 10 week old mice produced the greatest power, there was little difference in total cumulative work between 3 and 10 weeks (Fig 7.4.12). Cumulative work was reduced in EDL muscle from 30 week old mice and again further at 50 weeks (Fig 7.4.12). Diaphragm muscle from 3 week old mice produced the greatest cumulative work compared to all other age groups tested (Fig 7.4.13). Despite 10 week old diaphragm producing greater power than in 30 week old mice the total cumulative work was similar. Cumulative work was significantly reduced in diaphragm from 50 week old mice (Fig 7.4.13).

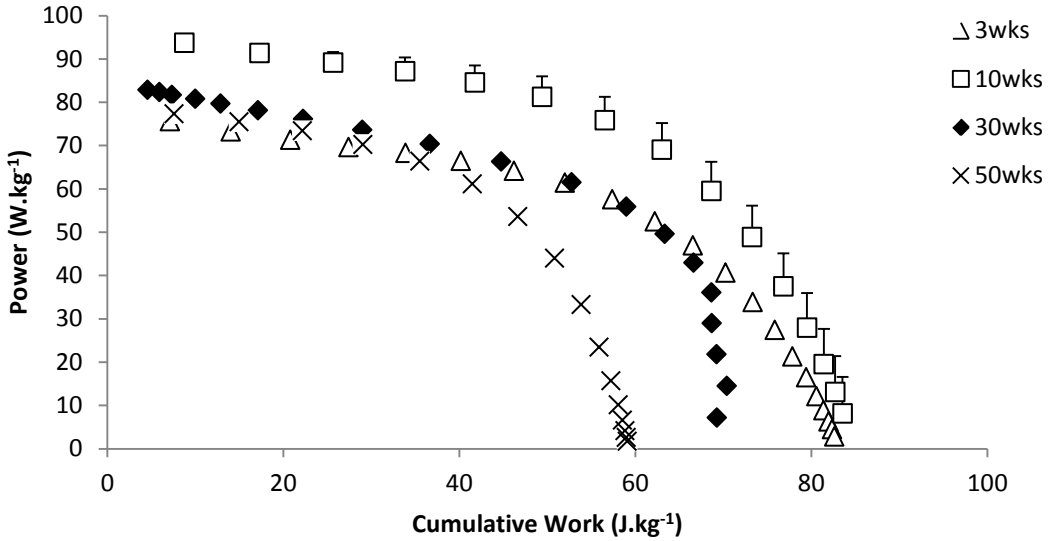


Figure 7.4.12. – The effect of increasing age on the relationship between power output and cumulative work in mouse EDL muscle. [Data represented as mean & SE for every second loop of the fatigue protocol; n = 10 in each case] Note, that for clarity, not all error bars have been added.

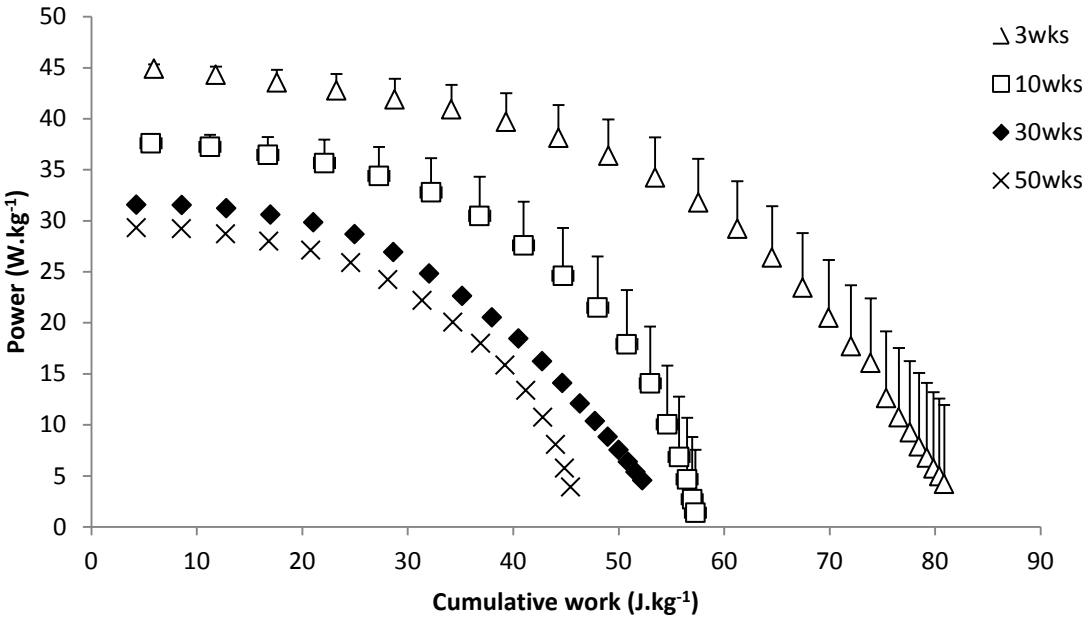


Figure 7.4.13. – The effect of increasing age on the relationship between power output and cumulative work in mouse diaphragm muscle. [Data represented as mean & SE for every second loop of the fatigue protocol; n = 10 in each case] Note, that for clarity, not all error bars have been added.

Recovery from Fatigue

For EDL, the 30-minute recovery period was sufficient to induce a significant increase in mean muscle power output over time. (Fig 7.4.14; ANOVA $p=0.002$). There was a significant effect of age on the recovery of muscle power output post fatigue (Fig 7.4.14; ANOVA $p<0.001$). The peak recovery over the 30-minute duration was 67%, 47%, 34% and 45% for 3, 10, 30 and 50-week groups respectively. Mean recovery of EDL at 3 weeks of age was significantly greater than that at 10, 30 and 50 weeks of age (Fig 7.4.14; Tukey $p<0.02$ in all cases). Recovery at 30 weeks of age was significantly reduced compared to 10 and 50 weeks of age (Fig 7.4.14; Tukey $p<0.001$). There was no significant difference in recovery between 10 and 50 weeks of age (Fig 7.4.14; Tukey $p=1$).

Peak recovery for diaphragm muscle occurred 10 minutes post completion of the fatigue run; there was no further subsequent recovery following this time (Fig 7.4.15; ANOVA $p=0.499$). The level of recovery was not significantly different between age groups (Fig 7.4.15; ANOVA $p=0.385$). Peak recovery was 89%, 93% 92% and 92% for 3, 10, 30 and 50 week groups respectively.

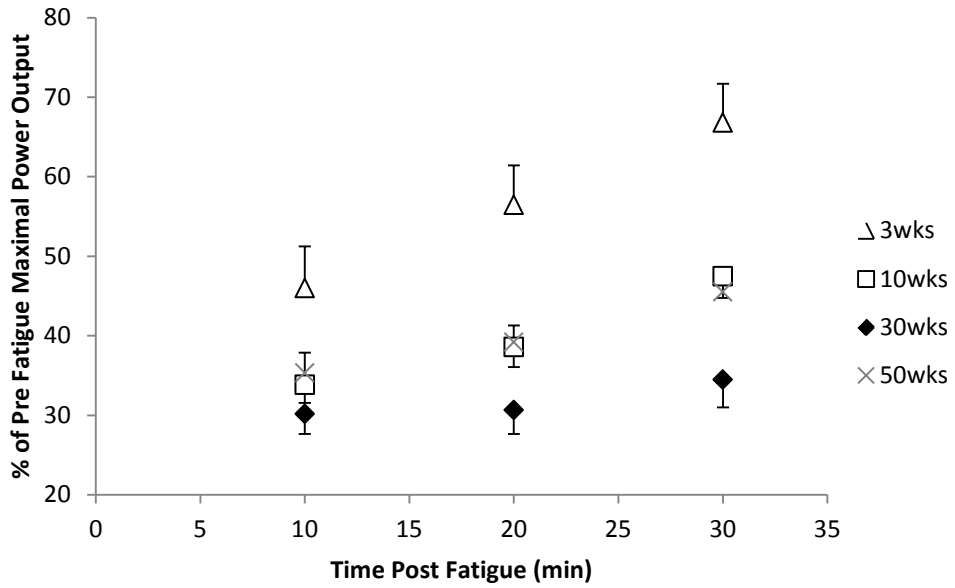


Figure 7.4.14 – The effect of age on mean recovery of power output from fatigue in mouse EDL [Data represented as mean ± SE: n=10 for 10 & 30 weeks; n=9 for 50 weeks; n=8 for 3 weeks; wks = weeks of age)

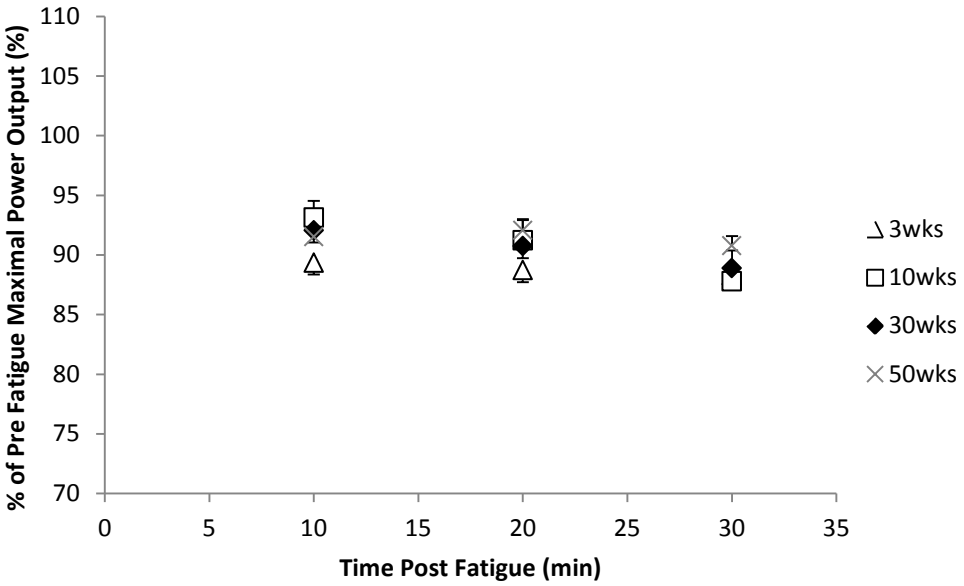


Figure 7.4.15 – The effect of age on mean recovery of power output from fatigue in mouse diaphragm [Data represented as mean ± SE: n=10 for 3, 10 & 30 weeks; n=9 for 50 weeks; wks = weeks of age)

7.5. Discussion

Effect of Age on Maximal Skeletal Muscle Force, Power Output, and Activation and Relaxation Times

From the age range used in the present study, muscles dissected from 10-week-old mice were deemed to be at physical maturity in terms of mechanical performance of skeletal muscle. For both EDL and diaphragm muscle 10-week-old mice produced the highest isometric stress, lowest activation and relaxation times, and highest power output. As was concluded by Chan and Head (2010), the present results indicate that the response of EDL muscle to ageing occurs independent of muscle atrophy as there was no age related reduction in muscle mass in this instance.

Development

EDL muscle from 3-week-old mice demonstrated lower maximal isometric stress and power, and increased activation time compared to the 10 week old. In contrast, there were no significant differences in maximal isometric stress, activation time, relaxation time, or work loop power in diaphragm muscle from 3-week-old mice compared to diaphragm from 10-week-old mice. Initially, this suggests that diaphragm muscle reaches its mature peak performance at a younger age than EDL and subsequently may underline the importance of the physiological function of breathing in comparison to locomotory performance in the early stages of life.

The reported differences between the tested muscles are likely to relate to the speed of development of the contractile properties. Skeletal muscle maximal force and the rate that this force is developed are largely related to the efficiency of the excitation contraction coupling process, and more specifically, the rate and quantity of SR Ca2+ release into the intramuscular space (Berchtold et al. 2000). It has been established that at birth the quantity of skeletal muscle SR is limited and occurs as only a loose network of tubes (Schiaffino & Margreth, 1969; Luff and Atwood, 1971). Luff and Atwood (1971) further demonstrated a fiber type specific increase in skeletal muscle SR reporting an increase from 1.1% fiber

volume at birth to 5.5% in adult EDL. Conversely, the SR content of soleus muscle (relatively slow) occupied 1.7% of the total muscle volume at birth, which increased to 2.9% in adult soleus. Luff and Atwood (1971) quantified the rate of SR development in these muscles, reporting that the majority of growth occurred in the initial 20 days and had reached adult values by 30 days in soleus and was still increasing after 60 days in EDL. This suggests that muscles with a predominantly faster phenotype at maturity take longer to develop the optimised process of excitation contraction coupling. The maturation of the SR Ca2+ release mechanism is related to age related shifts in predominant phenotype. Agbulut et al. (2003) demonstrated that after 21 days post gestation type IIb myosin heavy chain represented 54% of the total proportion of EDL, which increased to 87% at 90 days. As is widely recognised these fibers coincide with a greater normalised maximal force and power output and more rapid activation time due to enhanced contractile characteristics and glycolytic potential (Zierath & Hawley, 2004; Tallis et al. 2012; chapter 4). These findings help to explain the more rapid maturation of mechanical properties in diaphragm then EDL in the present study.

A further point to note is that maximal isometric stress and work loop power at 10 weeks was significantly greater (by 20% and 14% respectively) than the values at 8 weeks reported in chapter 4. This difference further highlights the speed of development in the muscle mechanical properties of the mouse.

Post Physical Maturity

After reaching maturity at 10 weeks of age, a relationship between increased age and reduced maximal isometric stress was evident in both EDL and diaphragm muscle. Interestingly the rate of decline in maximal stress was more greatly pronounced in diaphragm muscle. A reduction in maximal strength is a well-documented phenomenon in human studies and is supported by a reduction in isometric stress in previous in vitro research using older mammalian muscle isolations (Murray et al. 1980; 1985; Anianssonet al. 1986; Brooks & Faulkner, 1988; Zhang & Kelsen 1990).

In agreement with the findings of Chan and Head (2010), the present results indicate that an age related reduction in the mechanical performance of muscle may occur without associated atrophy. Interestingly, the muscle mass of EDL was significantly increased at 50 weeks of age and probably relates to a greater morphological size of the animal, with mice in this age range demonstrating an 83% increase in body mass when compared with 10 weeks of age. Evidence suggests that significant muscle atrophy only takes place in the final 20% of the animal's lifespan and subsequently the ages of mice in the present study potentially precede this (Brooks & Faulkner, 1988; Brown & Hasser, 1996; Pagala et al. 1998; Chan & Head, 2010). With the relatively small reduction in EDL muscle mass indicated in these studies, it is especially likely that atrophy is not the sole contributor to reduced muscle performance during early ageing.

The given reduction in the force generating capacity of EDL and diaphragm muscle is likely to relate to an age related decline in contractile function and fiber type composition.The age related reduction in functionality of excitation contraction coupling would greatly contribute to the loss of muscle function (Navarro et al. 2001). A decrease in the SR function associated with DHPR-Ryanodine Receptor uncoupling has been established; resulting in a reduction in the voltage gated SR Ca2+ release mechanism and consequently reduced Ca2+ availability at the contractile proteins (Larsson & Salviati 1989; Delbono et al. 1995; Renganathan et al. 1997). Furthermore the reported age related reduction in Ca2+ pump activity would correlate with the increase in relaxation time seen in EDL muscle in the present study (Larsson & Salviati 1989; Hunter et al. 1999; Narayanan et al. 1996).

Despite early work suggesting a higher age related decline in type II muscle fibers, it is now widely established that fiber number will decrease equally across all muscle fiber types (Lexell et al. 1988; Deschenes, 2004).A key aspect of the ageing phenotype appears to be a shift towards an oxidative metabolism with reduction in the cross sectional area of type II fibers being more pronounced (Aniansson et al. 1986; Alnaqeeb & Goldspink, 1986; Coggan et al. 1991). In the context of the present study, it is

likely that a slower muscle fiber type represents a greater proportion of the whole muscle cross sectional area and subsequently a reduced potential to produce high force.

Results of the present study fail to support research suggesting that the decline in muscle power output occurs more rapidly than the reduction in strength (Metter et al. 1997; Skelton et al. 1994; Deschenes, 2004). The magnitude of the reduction of maximal diaphragm power output was 23% compared to a 33% reduction in maximal isometric stress. Furthermore a significant reduction in muscle stress occurred at 30 weeks of age whilst maximal power output was maintained until 50 weeks of age. Despite a significant decrease in maximal EDL muscle stress production and elevated relaxation time, increasing age from 30 weeks to 50 weeks failed to have any significant effect on maximal muscle power output. This may suggest that the previously reported loss of power with age is a consequence of the further interaction of atrophy and CNS effects. The 13% decline in EDL power output between 10 and 50 weeks of age follows the same trend of that demonstrated in diaphragm (Fig 7.4.5); it is likely that this did not prove to be statistically significant due to the inter-individual variation in the data, possibly due to inter-individual differences in the rate of ageing.

With no significant change in actual muscle power output (W.kg-1) with age in EDL muscle, we decided to examine how this would relate to whole animal performance by normalising muscle power output to body mass (W.g-1). In this instance increasing age resulted in a significant reduction in power output in equal magnitude to that seen in maximal force. Even though the actual power output (W.kg-1) of EDL muscle varies little with increasing age, whole animal in vivo locomotory performance is likely to be significantly reduced. In essence, for 50 week old mice, EDL muscle would be acting to move almost double the body mass compared to that of 10 week old mice and subsequently the animal is likely to move at a reduced pace and fatigue quicker at the same relative intensity.

Effect of Age on Skeletal Muscle Fatigue

Results of the present study suggest that skeletal muscle fatigue resistance is affected by age and the pronunciation of the effects is muscle dependant. Despite these muscle dependent differences, a clear trend in the age related response has been established in the present study. Muscle in the development stage (3 weeks) has the greatest fatigue resistance; a significant reduction then occurs when the muscle reaches maturity (10 weeks). Following this, fatigue resistance significantly increases (30 weeks) before a second wave of reduced fatigue resistance with a further increase in age (50 weeks). This diverse and complex spectrum in the age related fatigue resistance response likely gives rise to the equivocal in vivo and in vitro results from studies that have previously examined this model (Lennmarken et al. 1985; Davies et al. 1986; Backman et al. 1995; Bemben et al. 1996; Lindstrom et al. 1997; Pagala et al. 1998; Izquierdo et al. 2001; Kent-Braun et al. 2002).

Cumulative Work

Cumulative work considers the total amount of work done by the muscle over the time course of the fatigue protocol and is related to the relationship between maximal work and fatigue resistance. During development cumulative work was greatest in 3 and 10 weeks old in EDL and is likely to be similar due to a greeter fatigue resistance at 3 weeks and subsequently a work can be produced over a longer duration. A similar principal can be applied for diaphragm muscle at 3 weeks where the greatest cumulative work was produced.

For 30 weeks cumulative work was reduced in EDL compared to 3 weeks, although power output was similar this reduction in cumulative work should be attributed to a faster time to fatigue in these muscles. This decrease occurs beyond a reduction in power below 50% and thus differences in time to fatigue between these groups are not shown in fig 7.4.8. Although producing a similar power output to EDL at 3 and 30 weeks, cumulative work for 50 week EDL was significantly reduced due to a reduced fatigue resistance. Similarly in diaphragm muscle although power output was reduced at 30 weeks, cumulative

work was similar to 10 weeks due to a greater fatigue resistance. Cumulative work was lowest at 50 weeks due to a reduced power and reduced fatigue resistance.

Development

In part, the complexity of these results may be explained by investigating the age related changes in muscle phenotype. Agbulut et al. (2003) quantified myosin heavy chain isoforms from birth to maturity in a number of skeletal muscles obtained from mice. At 21 days of age, EDL and diaphragm had a greater proportion of fibers with an oxidative capacity compared to mature levels. At this age EDL muscle was composed of 46% type I, IIa and IIx compared to these fibers making up 13% at 90 days. Consequently, type IIb fibers occupied the greatest percentage, which increased from 54% to 87% over this period. Although in diaphragm muscle from the same study there was little variation in the percentage of oxidative fibers (87% at 21 days compared to 94% at 90 days) unlike EDL neonatal myosin heavy chain was still present in diaphragm at 21 days. As demonstrated by Agbulut et al. (2003) it is likely that developing muscle from 3-week-old mice is composed of fibers with greater oxidative properties than at mature levels and subsequently show a greater resistance to fatigue.

With these potential differences in fiber type composition of developing diaphragm, the question beckons as to why maximal force, power, and activation and relaxation times are similar between 3 and 10-week muscle? Results by Agbulut et al. (2003) indicate that in 21-day-old diaphragm muscle, the higher level of neonatal fibers may be compensated by an increased type IIb fiber expression.

Post Physical Maturity

The current study is the first to provide evidence that rationalizes the previous equivocal data that examines the relationship between ageing and fatigue resistance. The present results indicate that at the skeletal muscle level, fatigue resistance is age and muscle dependent and likely to relate to predominant fiber type expression. In vitro evidence demonstrating an increased fatigue resistance via repetitive isometric contractions are likely to differ to those of the present study due to differences in the fatigue

mechanism promoted by this method (Gosselin et al. 1994; Pagala et al. 1998; Chan and Head, 2010). Unlike isometric measurements that primarily consider the decline in peak force, the work loop technique, as for in vivo power producing muscles, considers muscle force production over dynamic contractions accounting for the interaction of force production during shortening, resistance to muscle re-lengthening and changes in activation and relaxation time (Josephson, 1985). Subsequently, any age related changes in muscle activation and relaxation time, ability of the muscle to maintain force through shortening, and an increase in passive resistance to stretch will have profound additional effects on muscle fatigue resistance than the decline in force alone.

The augmented fatigue resistance at 30 weeks of age compared to maturity is likely to correlate with the age related shift to a more oxidative muscle fiber type (Aniansson et al. 1986; Alnaqeeb & Goldspink, 1986; Coggan et al. 1991). The progressive decrease in fatigue resistance at 50 weeks of age may therefore highlight the contribution of the previously discussed age related decline in muscle function. A further contributor to the given reduction in fatigue resistance may also relate to a potential age related increase in muscle collagen and fat content resulting in a larger non-contractile mass and subsequent muscle stiffness (Alnaqeeb et al. 1984; Kent-Braun et al. 2000; Martini et al. 2000). This increased resistance to stretch would amplify the proportion of negative work and offset the theoretical equation for maximal work loop power output (work loop power output = positive work – negative work: Josephson 1985). Unlike diaphragm muscle, EDL from 50-week-old mice fatigued significantly faster than EDL from 10-week-old mice, with such a finding likely to relate to the age related increase in relaxation time that is demonstrated in the work loop shapes examining the fatigue response. In this instance, the muscle will be active to a greater extent at the end of the shortening phase in each work loop thus increasing the eccentric work contribution and, as a consequence decreasing net work production. A further fatigue induced increase in relaxation time has been demonstrated and an accumulation of these effects will result in a further pronunciation in a faster fatigue time in the present study (Askew et al. 1997: Allen et al. 2008; Tallis et al. 2012; chapter 4). A relationship between time to fatigue and work loop shape has can also been seen. In general muscles that display a reduced fatigue resistance appear to have

an increased muscle relaxation time, thus negatively impacting the net work the muscle produces per cycle. Conversely, the ability of the muscle to maintain force through shortening throughout the fatigue shows little age related variation.

Effect of Ageing on Recovery from Fatigue

For diaphragm muscle, the level of peak recovery following the fatigue stimulation protocol was similar across all age ranges. Conversely, EDL muscle from 3-week-old mice recovered to the greatest degree and recovery at 30 weeks of age was significantly reduced.

Although the acute response of the contractile properties following muscular fatigue in the aged population has received little attention, particularly in vitro, human and animal evidence suggests that recovery is largely unaffected. Allman and Rice (2001) failed to demonstrate a difference in the degree of low-frequency fatigue between young and elderly human subjects following repeated contractions at 60% of maximal voluntary contraction. Likewise, Gonzalez and Delbobo (2001) concluded that despite changes in maximal tetanic stress of mouse EDL and soleus muscle with increasing age, recovery time and stress production following fatigue via repetitive isometric contractions were unaffected by age.

Previous findings using the work loop technique have demonstrated that the recovery of power output occurs faster in muscle with a slower fiber type (Tallis et al. 2013; chapter 5). Consequently this may explain why EDL muscle from 3-week-old mice recovered quicker in the present study.

Practical Implications of the Study

With the populations of most western nations growing older and the related increase in healthcare costs, having a greater understanding of the ageing response is important in the potential development of innovations to improve human health and quality of life (Deschenes, 2004). Initially the study highlights significant reductions in skeletal muscle performance that occurs at a relatively young age. Early ageing was associated with a greater loss of diaphragm force and power compared to locomotory EDL muscle. Subsequently highlighting the importance of the development of effective interventions to improve the

performance of diaphragm muscle in relatively young populations, given its importance to cardiorespiratory function. Furthermore chronic obstructive pulmonary disease (COPD) is associated with reduced respiratory muscle strength and this coupled with the ageing effect demonstrated in the present findings may contribute to the severity of the symptoms observed in elderly patients (Polkey et al. 1996; Ottenheijm et al. 2008).

With falls causing greater rates of morbidity and mortality in elderly citizens, the importance of understanding the ageing effects on peripheral muscle performance is of equal importance (Aschkenasy & Rothenhaus, 2006). It has been demonstrated that impaired muscular endurance contributes to the instance of falls among the elderly (Schwendner et al. 1997).

Subsequently the reduction in maximal skeletal muscle force and substantial decrease in fatigue resistance, as shown in relatively young EDL muscle in the current findings, is likely to be further accelerated in an older population and would greatly contribute to this phenomenon if transferable to humans.The present study further indicates that the reduction in locomotory performance in vivo may be additionally pronounced when muscle function is related to whole animal body mass. This highlights an important interaction between muscle weakness and body mass, where increasing body mass would accelerate the age related reduction in whole animal locomtory performance. At 50 weeks of age two distinct groups in whole animal body mass were evident (Fig 7.4.1). Further research should be conducted to establish the relationship between skeletal muscle performance, increasing age and adipose tissue in order to further our understanding into the interaction between ageing and obesity. A further area of interest would be to examine the effect of increased physical activity to offset the demonstrated age related decline in direct muscle performance. This is explored in greater depth in the future work section of the thesis (chapter 10).

The present study furthers our understanding of the relationship between ageing and the direct effect on muscle function. However, the suggested age related increase in central fatigue that occurs in endurance tasks may potentially magnify the ageing response seen in the present study when relating these results

to in vivo performance (Bilodeau et al. 2001; Deschenes, 2004). Furthermore the present research is conducted using female mice and although the trends demonstrated in the present study are unlikely to change, the time course and magnitude of the ageing response is likely to differ in male mice and is liable to relate to sex related differences in hormone secretion (Moran et al. 2005; Chan & Head, 2010).

Conclusion

The present study demonstrates a muscle specific change in skeletal muscle mechanical properties at different ages. Diaphragm muscle appears to reach physiological maturity at a faster rate than EDL, which highlights selective rapid development for cardiorespiratory function when compared to locomotory performance. Although muscle specific, the present findings infer that increasing age beyond physical maturity is associated with a reduction in the mechanical performance of skeletal muscle. The study uses mice much younger than those in previous work and still demonstrates significant reduction in muscle contractility. In contrast to in vivo evidence, the reduction in maximal force was more greatly pronounced than the loss of power output in both of the investigated muscles. This subsequently suggests that the greater reduction in muscle power in human research is a consequence of the further interaction of atrophy and neuromuscular innervation. Interestingly, the loss of maximal muscle force and power were greater in diaphragm muscle compared to EDL. In contrast however, in the oldest age group, fatigue resistance of EDL was significantly impaired. Furthermore findings of the present research may help to rationalise the previous equivocal data examining the effects of age on muscle fatigue. Again although the magnitude of the response was muscle specific, a distinct pattern in the change of fatigue resistance has been identified. Without a demonstrated reduction in EDL muscle mass, the present results indicate that the age related decline in skeletal muscle performance occurs prior to associated atrophy mechanisms. Therefore it is likely that the reduction in skeletal muscle function demonstrated in early ageing is a result of a combination of a shift to a more oxidative fiber type and age related deterioration in contractile performance.

8. Does the Ergogenic Benefit of Caffeine Change with Age? The Effect of Physiological Concentrations of Caffeine on the Power Output of Maximally Stimulated Mouse EDL and Diaphragm Muscle

8.1. Abstract

The effects of caffeine as an ergogenic aid have been widely studied in mature human populations with well-established improvements during endurance and high intensity exercise. However, the potential age related ergogenic response of caffeine has received less attention and could be an important area of investigation due to age related changes in muscle composition and contractility. The present study aims to build upon previous work using the work loop technique to assess the direct effect of 70μM caffeine (physiological maximum) on the maximal power output of isolated mouse EDL and diaphragm muscle from four different age groups (3, 10, 30 & 50 weeks). In both mouse EDL and diaphragm muscle 70μM caffeine treatment resulted in a significant increase in maximal muscle power output that was generally greatest at 10 and 30 weeks (up to 5% & 6% improvement respectively). This potentiation of maximal muscle power output was significantly lower at 50 weeks (up to 3% & 2% improvement for EDL and diaphragm respectively) and in mice in the development stage at 3 weeks (up to 1% & 2% improvement for EDL and diaphragm respectively).The previously established muscle specific caffeine response appears to be prevalent in skeletal muscle but is likely affected by both muscle development and the age related decline in muscle contractility. Consequently, caffeine could be used to enhance muscle force in a more senior population; however this established effect may be significantly reduced when compared to skeletal muscle from younger individuals.

Key Words: Ageing, Ergogenic Aid, Sarcopenia, Skeletal Muscle, Work Loop,

8.2 Introduction

The ergogenic benefit of caffeine to promote performance enhancing effects has been extensively studied, and is well recognised to induce increases in endurance, strength and power activities (Graham *et al.* 2001; Davis & Green, 2009).

The majority of research evaluating the ergogenic effects of caffeine has been conducted on subjects within the range of physiological maturity. The benefit of supplementation in a more senior population has received very little attention. We have previously demonstrated (Chapter 7) a muscle specific change in contractility with age, which is likely to affect the direct ergogenic response of caffeine. Associated age related changes in fiber type composition and the efficiency of the excitation-contraction coupling process that have previously been demonstrated as factors influencing the performance enhancing affect of caffeine support this notion (Delbono *et al.* 1995; Navarro *et al.* 2001; Deschenes, 2004; Tallis *et al.* 2012; chapter 4).

It is believed that caffeine in older adults is metabolised in the same way, however a given dose may represent a greater plasma concentration due to the age related changes in body composition resulting in an increased adipose tissue to lean body mass ratio (Massey, 1998). Rees *et al.* (1999) investigated the central effects of 250mg caffeine supplementation 50-65 year olds compared with a younger 20-25 year old group. Age was associated with a significant reduction in psychomotor and cognitive performance, and as with the young subjects, caffeine was effective at increasing both psychomotor and cognitive performance, particularly by offsetting the decline in performance overtime. Swift and Tiplady (1988) further suggested that an older population may be more sensitive to the effects of caffeine at the psychomotor level, with these subjects showing greater improvements in attention and choice reaction time compared to younger participants.

Research by Norager *et al.* (2005) appears to be the only study to assess the effects of caffeine supplementation on exercise performance in an elderly population. The study reported significant

increases in cycling endurance (25%) and arm flexion endurance (54%), coupled with a reduction in RPE in men and women aged over 70, following 6mg/kg^{-1} caffeine consumption. No significant differences were found in muscle strength, walking speed, and reaction time. Norager *et al.* (2005) recognised that the reported increase in endurance may be due to the effect of caffeine on psychological ability, as it was stated that the elderly are capable of working at higher loads for prolonged periods than that achieved in their study. Norager *et al.* (2005) further noted that the increase in arm flexion was markedly higher than that seen in an earlier study on younger individuals. As with young adults, Pincomb *et al.* (1985) and Conard *et al.* (1982) reported that caffeine supplementation elicited significant increases in blood pressure but with no concurrent positive inotropic effect in elderly men. Furthermore, the ergogenic effect of caffeine on exercise performance has not been studied in children and adolescents despite the high consumption of caffeinated products in this population (American Academy of Paediatrics, 2011). In summary the current literature indicates very little about the age related effects of caffeine on physical performance. Measurement of this potential effect using direct *in vitro* testing methods will allow a more accurate investigation by eliminating complications in population plasma concentrations that would occur in whole body studies due to the earlier highlighted changes in adipose tissue to lean body mass ratio (Massey, 1998).

In vitro research has confirmed the effect of caffeine via direct potentiation of isolated skeletal muscle performance at high (mM) (Allen and Westerblad, 1995), and more recently, at human physiological concentrations (50-70μM) (James *et al.* 2004; Tallis *et al.* 2012; chapter 4). The mechanism of action for caffeine in this instance is attributed to its adenosine receptor antagonistic properties specifically at the A1 receptors on the skeletal muscle membrane and/or its ability to bind to ryanodine receptors (RYR) of the sarcoplasmic reticulum (SR) (Damiani *et al.* 1996; Fredholm 1999; Rossi *et al.* 2001). The net effect is modified excitation contraction coupling specifically through enhanced ion handling (Graham, 2001; Kalmar & Cafarelli, 2004). Skeletal muscle ageing is associated with a reduction in excitation contraction coupling and reduced SR Ca^{2+}release as a mechanism for the related loss of muscle force (Delbono *et al.*

1995; Navarro *et al.* 2001). These age related changes may significantly affect the skeletal muscle response to caffeine treatment.

The present study aims to build on the previous findings of Tallis *et al.* (2012; chapter 4) by being the first to assess the effect of the maximal physiological concentration of caffeine (70μM maximal) on the maximal power output of isolated mouse EDL (predominantly fast-twitch) and diaphragm muscle (mixed muscle fiber type) during development (3 weeks old) and at various stages post physiological maturity (30 & 50 weeks old). As previously discussed in chapter 7, 50 weeks represents 'middle age' and significant reductions in muscle contractility were reported deeming this an appropriate age range to examine the age related ergogenic effect of caffeine. The research uniquely uses the work loop technique to approximate the cyclical length changes used by some muscles during *in vivo* muscle performance and is the first (*in vivo* or *in vitro*) to assess if the ergogenic effect of caffeine is altered by the development and ageing of skeletal muscle. Tallis *et al.* (2012; chapter 4) further demonstrated a fiber type specific response to caffeine, therefore the present study will also asses the age related ergogenic effect of caffeine on two different skeletal muscles with diverse function and different fiber type composition. In light of our previous findings it is suggested that the direct treatment of mouse EDL and diaphragm with 70μM caffeine will cause an age dependant improvement in muscle power output. Furthermore the ergogenic response will be muscle specific and will relate to differences in the stages of muscle development.

8.3 Methods

A more detailed account of the methods is given in the general methods section (chapter 3).

From birth mice were housed in groups of 8 without access to running wheels and aged to 3 weeks, 10 weeks, 30 weeks, and 50 weeks (*n* = 20 for each age group) before sampling. Rational for the use of mice at these ages has been previously discussed in chapter 7. 3-week-old mice were used to represent muscle in a developmental stage; 10 week old represents physical maturity, 50 week old was used to represent 'middle-aged' muscle. Mice from each age range were tested within 7 days of reaching their target age. Ageing was associated with significant weight gain in the mice used in the present study.

Either whole diaphragm (approximate composition: 6.2% IIb, 34.6% IIx, 43.6% IIa, 15.6% I, Agbulut *et al.* 2003) or both EDL (approximate composition: 86.8% type IIb, 9.3% IIx, 3.9% I, Agbulut *et al.* 2003) muscles were dissected from each mouse (body mass (g): 3 weeks = 15.8± 1.2; 10 weeks = 31.9±0.8; 30 weeks = 41.6±0.8; 50 weeks 58.2±5.4; n=20 in each case).

EDL of the left hind limb, or the right half of the diaphragm, was immediately frozen in liquid nitrogen and the opposing EDL muscle or section of diaphragm prepared for mechanical assessment. Muscles were prepared as previously described in the methods section of chapter 7.

Once dissected the muscle was placed in the muscle bath and circulated with oxygenated Krebs solution at 37^{o}C.The muscle length and stimulation amplitude (14-18V for EDL; 10-16V for diaphragm) were optimised to produce maximal twitch force. Following this each muscle was subjected to a 250ms burst of electrical stimulation in order to produce a tetanus response. Stimulation frequency was optimised in order to produce maximal tetanus force (usually 200Hz for EDL; 140Hz for diaphragm, and this did not change with age). A 5-minute rest period was imposed between each tetanus in order to allow the muscle sufficient recovery between stimulations.

The Work Loop Technique

Each muscle was subjected to four sinusoidal length change cycles per set at a total symmetrical strain of 0.10. A cycle frequency of 10Hz and 7Hz was used for EDL and diaphragm muscle respectively. 10Hz represents the cycle frequency that has previously been shown to elicit maximal power output in EDL (James *et al.* 1995). 7Hz represents the cycle frequency that elicited maximal power output in diaphragm in preliminary work by the authors and is similar to previous findings (Altringham and Young, 1991). These frequencies have been demonstrated to be optimal for maximum power generation in adult mice but may not be uniform across all age ranges. However, the present study allows a direct comparison across age ranges at the selected cycle frequencies. Usually 49ms burst duration was used for EDL which is in keeping with that previously used at 10Hz cycle frequency (James *et al.* 1995). The burst duration commonly used to elicit maximum power output in diaphragm muscle was 55ms. On occasions the burst duration had to be increased or decreased to adjust the number of stimuli given. A stimulation phase shift of -2 ms and -5 ms were used for EDL and diaphragm respectively as they corresponded with maximal power output.

The Effect of 70µM Caffeine Treatment

Each muscle was subjected to a set of four work loop cycles every ten minutes over a 130-minute duration (James *et al.* 2005; Tallis *et al.* 2012; chapter 4). Three measurements of the muscle's maximal power output were made in standard Krebs-Henseleit solution and this formed the control. Following this the circulating fluid was changed to Krebs containing 70µM caffeine and a further 6 measurements of maximal power were made. The assessment concluded with a washout period were the circulating fluid was changed back to standard Krebs and a further four measurements of maximal power were taken (Fig 7.3.1).

At the end of the experiment the muscle was detached from the rig, tendons removed, then weighed in order to calculate isometric stress ($kN.m^{-2}$) and normalised muscle power ($W.kg^{1}$).

Statistical Analysis of the Data

In control conditions muscle power output will decrease over time due to the gradual development of an anoxic core (Barclay, 2005). Over the 130-minute duration of the protocol used in the present study, muscle power had decreased to 92.5±0.61% of initial power output. In order to avoid the given deterioration in muscle power output masking the effects of the caffeine treatment, a 1st order regression equation was calculated using the pre-treatment control data and post treatment washout control data to identify the linear relationship between muscle power output and time. This regression equation was then used to determine theoretical control muscle power output for each time point during caffeine treatment (James *et al.* 2005; Tallis *et al.* 2012; chapter 4).

Initially for each treatment group, pre-treatment controls were compared directly against post treatment washout controls using a paired t-test. There was no significant difference between these measurements (Paired T-test $p>0.7$ in each individual case) therefore these results were pooled to form controls. Thereafter, it was assumed that any subsequent change in muscle power output was solely the effect of the given caffeine treatment. Consequently, controls were compared directly against caffeine treatment using a further paired T-test for each treatment group. To establish whether a difference in the effect of caffeine occurred between ages a single factor ANOVA was conducted on the caffeine data for each of EDL and diaphragm muscle. When the ANOVA indicated a significant difference between age groups, Tukey post hoc tests were used to identify where these differences occurred. An independent samples t-test was conducted to compare the caffeine treatment results for EDL and diaphragm of the same age group in order to identify whether the ergogenic benefit was greater in a particular muscle each age.

Results were interpreted as significant when $p< 0.05$. Values are displayed as mean ± standard error.

8.4. Results

Whole Animal Body Mass

Increasing age resulted in a significant increase in mean mouse body mass and upon visual inspection and dissection it was evident that this was due to an increase in adipose tissue (ANOVA $p<0.001$). At 50 weeks of age mean body mass had increased by 73% compared to that at 3 weeks. At 50 weeks individual body masses had either increased above 70g or appeared to stay below 50g, which is similar to the mean body mass at 30 weeks. This information has been previously reported in chapter 7 where it is discussed in greater depth.

The Effect of 70μM Caffeine Treatment on the Maximal Power Output of Mouse EDL

Treatment of mouse EDL with 70μM caffeine resulted in a significant increase in power output by up to 1%, 4%, 5% and 3% for 3, 10, 30 and 50 week old mice respectively (Fig 8.4.1; paired t-test$p<0.001$ between control and caffeine power output in all cases). There was a significant effect of age on the caffeine-induced improvement in power output (Fig 8.4.1; ANOVA $p<0.001$). The caffeine induced increase in muscle PO was the highest at 30 weeks and was significantly greater than the response at 3 and 50 weeks (Fig 8.4.1; Tukey $p<0.001$ in both cases) and had a tendency to be greater than that at 10 weeks (Fig 8.4.1; Tukey $p=0.079$). The ergogenic benefit at 3 weeks was significantly lower than at all other ages (Fig 8.4.1; Tukey $p<0.005$ in all cases). The increase in muscle PO at 10 weeks did not prove to be significantly different to that at 50 weeks (Fig 8.4.1; Tukey $p=0.733$).

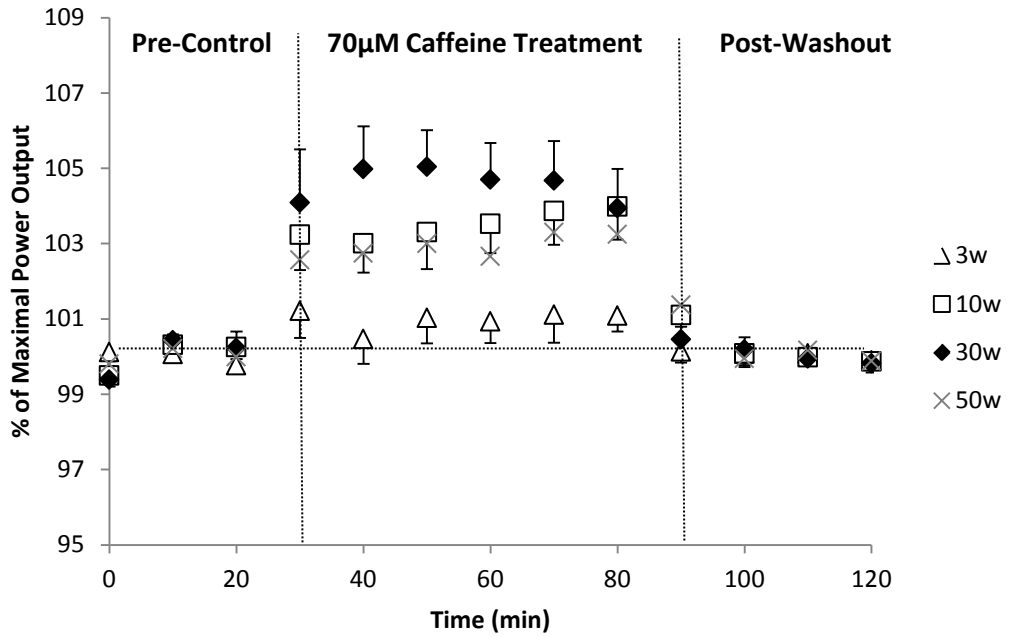


Figure 8.4.1 – The effect of 70µM caffeine on the mean acute maximal power output of mouse EDL isolated from 3, 10, 30 and 50 week old mice [Data represented as mean ± SE: n=10 in each case].

The Effect of 70µM Caffeine Treatment on the Maximal Power Output of Mouse Diaphragm

Treatment of mouse diaphragm with 70µM caffeine resulted in a significant increase in power output by up to 2%, 6%, 4%, and 2% for 3, 10, 30 and 50 week old mice respectively (Fig 8.4.2; paired t-test$p<0.001$ in all cases). There was a significant effect of age on the caffeine-induced increase in power output (Fig 8.4.2; ANOVA $p<0.005$). The caffeine induced increase in power output at 10 weeks was the highest and was significantly greater than at 3, 30 and 50 weeks (Fig 8.4.2; Tukey $p<0.004$ in all cases). Power output was also significantly higher at 30 weeks compared to at 3 weeks and 50 weeks (Fig 8.4.2; Tukey $p<0.02$ in both cases). There was no significant difference in power output between 3 weeks and 50 weeks (Fig 8.4.2; ANOVA Tukey $p=0.864$).

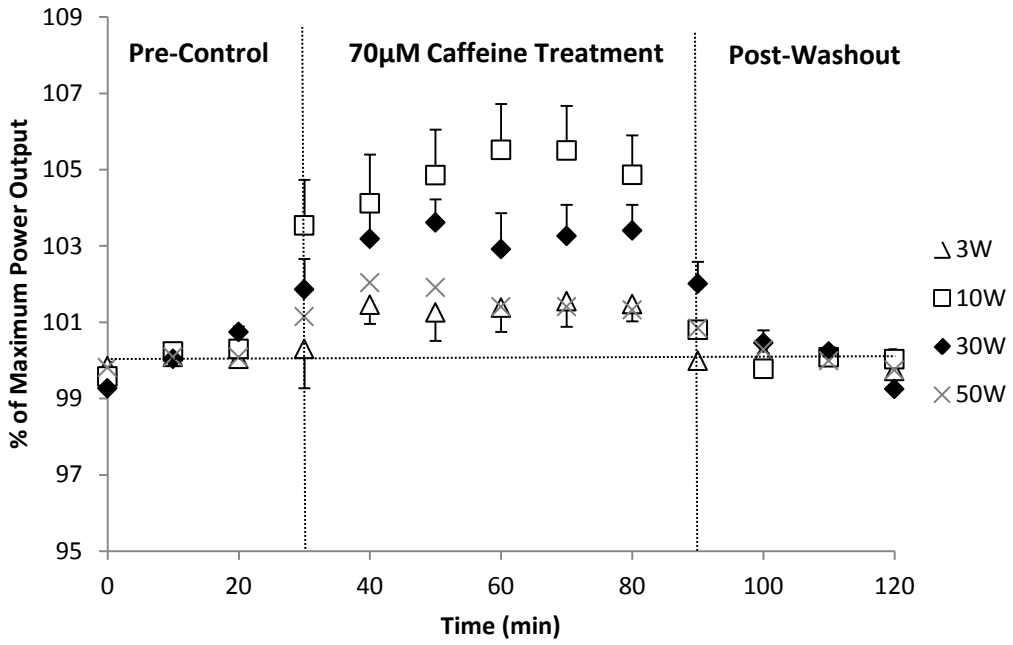


Figure 8.4.2 – The effect of 70µM caffeine on the mean acute maximal power output of mouse diaphragm isolated from 3, 10, 30 and 50 week old mice [Data represented as mean ± SE: n=10 in each case].

The ergogenic benefit of 70µM caffeine treatment was significantly greater in EDL muscle compared to diaphragm at 30 and 50 weeks (two-sample t-test$p<0.005$ in both cases). The caffeine-induced potentiation of diaphragm power output had a tendency to be greater than EDL at 10 weeks (two-sample t-test$p=0.054$). The caffeine induced increase in maximal muscle power output at 3 weeks was not significantly different between muscles (independent samplest-test$p=0.54$). The peak effect of caffeine over this age range for EDL and diaphragm is displayed in figure 8.4.3.

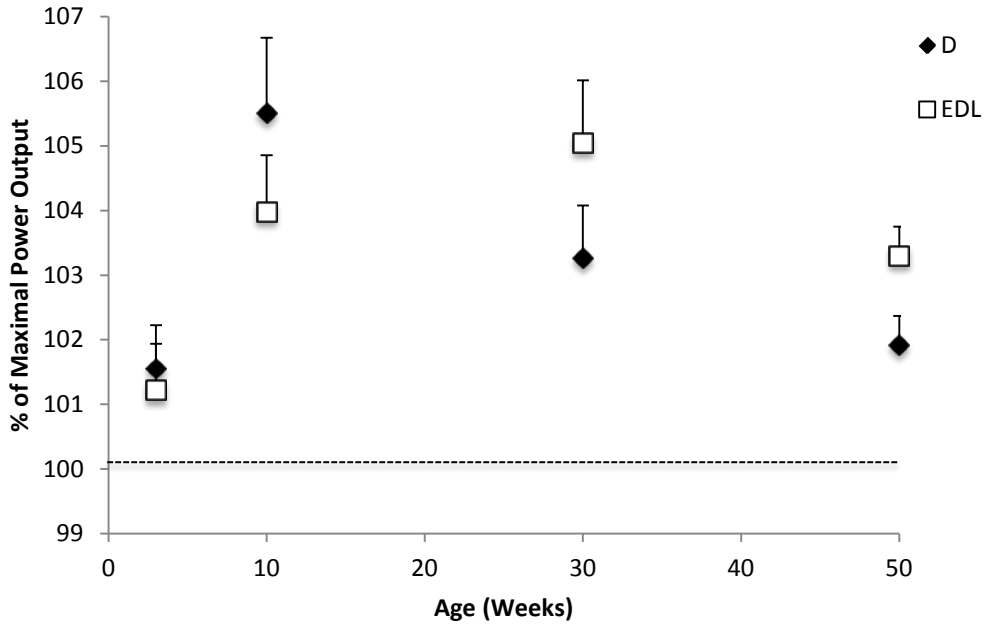


Figure 8.4.3 – The comparison of the peak effect of caffeine on muscle power output with increased age between EDL and diaphragm [Data represented as mean ± SE: n=10 in each case].

In order to assess if body mass, or more specifically the potential associated increase in muscle fat content, affected the direct ergogenic effect of caffeine, the caffeine induced increase in net power output was compared between the two distinct groups highlighted at 50 weeks in Figure 7.4.1. Whole animal body mass of the mice where diaphragm preparations were taken displayed and equal divide (n=5 in each case) in those below 50g (43±2g; mean±SE) and those greater than 80g (90±3g; mean±SE). Subsequently the caffeine response was compared between these groups and no significant difference in the effect was shown (Fig 8.4.4; two-sample t-testp=0.45).

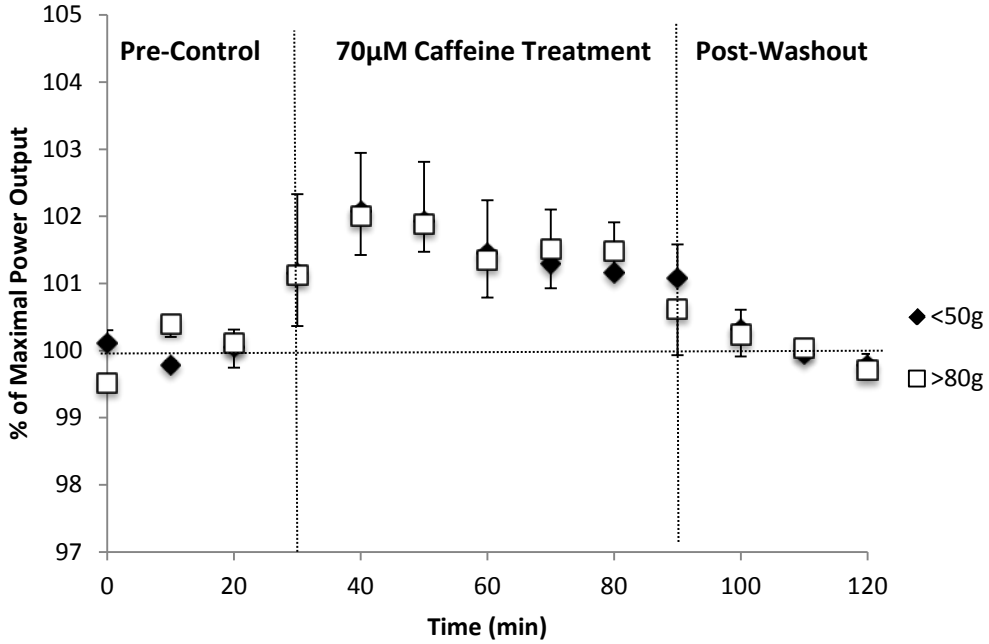


Figure 8.4.4 – The effect of high (90g) and low (43g) whole animal body mass on the direct ergogenic benefit of 70µM caffeine on 50-week-old diaphragm muscle [Data represented as mean ± SE: n=5 in each case].

8.5. Discussion

The Direct Effect of 70μM Caffeine on Post Mature Skeletal Muscle

The direct treatment of mouse EDL and diaphragm muscle with 70μM caffeine significantly potentiated maximal power output independent of age. Interestingly, the present findings indicate a muscle specific relationship between age and level of direct ergogenic effect. The caffeine-induced potentiation of maximal muscle power output was greatest at 30 weeks for EDL but was significantly reduced in the oldest age group. The peak ergogenic benefit in diaphragm muscle occurred at 10 weeks which reduced with increased age. The caffeine induced increase in EDL muscle power output at 10 weeks (up to 4%) is consistent with the 3% reported by James *et al.* (2005) and Tallis *et al.* (2012; chapter 4) using similar experimental methods. This study is the first to assess the direct effect of 70μM caffeine on diaphragm muscle and the 6% increase in muscle power is comparable to that previously reported for mouse soleus (Tallis *et al.* 2012; chapter 4). The given age related reduction in the caffeine induced force potentiation, identified in the present study, is likely to correlate with the age related decline in muscle function (Chapter 7) suggesting that the mechanism of ageing may be accountable for this loss in the ergogenic effect.

The rate of muscle activation and force production is primarily determined by effectiveness of excitation contraction coupling and the intramuscular Ca^{2+} transient time (Berchtold *et al.* 2000). The demonstrated caffeine induced increase in maximal power output is likely to occur due to the effects of caffeine as a modifier of excitation-contraction coupling (Graham, 2001; Kalmar & Cafarelli, 2004). Mechanistically, it is believed that caffeine acts as a direct agonist to adenosine receptors on the skeletal muscle membrane and has been demonstrated to bind to ryanodine receptors (RyR) of the sarcoplasmic reticulum (SR) (Bhat *et al.* 1997; Damiani *et al.* 1996; Fredholm *et al.* 1999; Rossi *et al.* 2001). It is likely that these actions then result in an improved opening of RyR channels promoting a greater Ca^{2+} release into the intracellular space, an increased Ca^{2+}myofibrillar sensitivity, a decreased SR Ca^{2+} pump sensitivity and increased SR Ca^{2+} permeability. Consequently, a reduction in the rate of Ca^{2+} efflux from the intracellular space back to

the SR is likely to occur resulting in an elevated basal and subsequently activated intracellular Ca^{2+} concentration (Allen *et al.* 1989; Allen & Westerblad, 1995).

Ageing skeletal muscle is associated with a reduction in the functionality of excitation-contraction coupling; subsequently this will result in a reduction in contractile performance and potentially the sensitivity to caffeine (Navarro *et al.* 2001). Renganathan *et al.* (1997) demonstrated a significant age related reduction in SRCa^{2+} release of rat EDL and soleus muscle attributed to DHPR-RyR uncoupling. This was further supported by Delbono *et al.* (1995) who confirmed a similar effect in fast fibers of human quadriceps muscle. A reduction in the voltage gated SR Ca^{2+} release mechanism would result in a decreased Ca^{2+} availability for the contractile proteins and consequently a reduction in contractile force. Consequently, in the context of the present study, caffeine may still bind to RyR in the older aged group muscle, but a reduction in the number of physiologically active RyR will result in a decrease in the force and power enhancing effects of caffeine. In addition Larsson & Salviati (1989) reported an age related reduction in SR Ca^{2+} concentration and Ca^{2+} pump activity of fast twitch muscle of chemically skinned rats. Therefore, it is likely that the reduction in the ergogenic properties of caffeine in post mature muscle arise from a reduced ability to increase SR Ca^{2+} release. Hunter *et al.* (1999) and Narayanan *et al.* (1996) provide further support for a significant reduction in SR Ca^{2+} uptake and Ca^{2+}-ATPase activity in older muscle. A potential shift in the primary mechanism of caffeine acting in aged skeletal muscle may occur if this effect is coupled with the caffeine induced decrease in SR Ca^{2+} pump sensitivity as reported by Allen *et al.* (1989) and Allen and Westerblad (1995). In this instance there will be a further increase in basal and post contractile intracellular Ca^{2+} concentration eliciting a greater force.

The Direct Effect of 70µM Caffeine on Developing Skeletal Muscle

Despite the regular consumption of caffeine containing products by children and caffeine containing energy drinks marketed specifically at this age group, the effect of caffeine on exercise performance has not been fully validated in younger people (American Academy of Paediatrics, 2011). Turley, Bland and Evans (2008) reported limited effects of low, mild and high dose caffeine (1, 3 and 5 $mg.kg^{-1}$) on the

physiological responses to exercise in 7-9 year old children. Caffeine treatment had no effect on substrate metabolism; however, mild and moderate dose caffeine treatment resulted in a slight increase in blood pressure and slight decrease in heart rate.

The present study shows that the treatment of muscle from 3-week-old mice with 70µM caffeine has only a small, but significant, effect on the potentiation of maximal work loop power output. The ergogenic benefit in this age group was not significantly different between diaphragm and EDL and the given response is likely to relate to the stage of postnatal muscle development. Schiaffino and Margreth (1969) and Luff and Atwood (1971) established that at birth, skeletal muscle SR is sparse and occurs as a loose network of tubes. Furthermore, Luff and Atwood (1971) demonstrated a muscle fiber type specific increase in mouse SR from 1.1% of fiber volume at birth to 5.5% in adult EDL (predominantly fast-twitch), and from 1.7% at birth to 2.9% in adult in soleus (predominantly slow-twitch). SR development occurred most rapidly in the initial 20 days and had reached adult values by 30 days in soleus and was still increasing after 60 days in EDL. Therefore, it should be considered that in the 3-week-old diaphragm and EDL muscle used in present study, SR is not fully developed, thereby limiting the action of caffeine on RYR and consequently reducing the level of caffeine induced Ca^{2+} release and subsequent force potentiating effects.

Previous literature and the present research suggest that caffeine in a young age group has both a limited effect on physiological responses to exercise and a limited direct effect on skeletal muscle. However, the stimulant effects at the central nervous system should be fully evaluated before conclusions are made about the value of caffeine as an ergogenic aid in children. The effectiveness of caffeine on the cognitive function of children requires further investigation; Hughes and Hale (1998) reported only slight improvements in vigilance performance and decrease reaction time in this population. It is unlikely however that caffeine supplementation in a young age group will greatly enhance sporting performance and the detrimental effects of high dose and regularly consumed caffeine in this population, reported in

studies by Hughes and Hale (1998) and a review by American Academy of Paediatrics (2011), potentially outweigh its benefit.

The Muscle Specific Action of 70μM Caffeine Treatment

At 10 weeks the caffeine induced potentiation of maximal skeletal muscle power output was significantly greater in diaphragm muscle compared to EDL. However, with a further increase in age the effect of caffeine was greater in more aged EDL. It has been firmly established that the force potentiating effects of caffeine are more greatly pronounced in muscle with a predominantly slower fiber type (Fryer & Neering, 1989; Rossi *et al.* 2001; Tallis *et al.* 2012; chapter 4). Although of mixed muscle phenotype, diaphragm will consist of a significantly greater proportion of oxidative muscle fibers than EDL and subsequently the caffeine-induced benefit was greater in this muscle during maturity. In order to establish why this muscle specific action of caffeine changed, the age related alterations in phenotype should be considered. Despite early work suggesting that muscle fiber number decreases more rapidly in type II fibers it is now widely established that muscle fiber number will decrease equally across fast and slow fiber types (Deschenes, 2004).A key aspect of ageing phenotype appears to be a shift towards an oxidative metabolism as the reduction in the cross-sectional area (CSA) of type IIb fibers appears to be more pronounced (Aniansson *et al.* 1986; Coggan *et al.* 1991).Alnaqeeb and Goldspink (1986) showed a significant 22.7% reduction in the CSA of fast glycolytic (FG) fibers of rat EDL, the CSA of fast-oxidative glycolytic (FOG) fibers increased by 12.4% in conjunction with a relatively small increase in slow oxidative (SO) CSA. The ageing of mice to 30 weeks in the present study is likely to evoke this specific CSA phenotype shift in EDL and an increase in the CSA of SO may explain the elevated benefit of caffeine at this age group. Any further increase at 50 weeks is probably masked by the previously discussed deterioration of excitation contraction coupling.

The Direct Effect of 70μM Caffeine on Mouse Diaphragm Muscle

Kivity *et al.* (1990) demonstrated that high dose caffeine significantly improved the pre and post exercise forced expiratory volume in one second (FEV1) in healthy human male subjects. Although this effect was largely attributed to the bronchodilator properties of caffeine, thought should also be given to a potentially increased performance of the respiratory muscle. The present study is the first to show a direct effect of physiological concentrations of caffeine to potentiate the maximal PO of diaphragm muscle. This extends the work of Brinbaum and Herbst (2004) who also suggested that caffeine may provide a modest ergogenic benefit by increasing respiratory efficiency.

Practical Implications and Limitations of the Present Findings

The present findings infer that caffeine can be used to directly improve skeletal muscle performance in mice across a broad age range. However, when applying these results to human performance it should be considered that there would be differences in caffeine metabolism between species e.g. caffeine has a shorter half-life in rodents (Fredholm *et al.* 1999). The development and ageing of skeletal muscle over time will differ greatly between mice and humans making it difficult to give a human equivalent of the ages used in the present study. That said the age-associated changes in the mechanical properties of mouse muscles follow the typical pattern of human ageing suggesting that the effect of caffeine treatment in the present study is likely to be similar.

The mechanisms proposed in the present study are likely to significantly contribute to the varied force potentiating effect of caffeine treatment with age, however, further research assessing the action of caffeine on increased Ca^{2+} sensitivity of the myofibrils and decreased SR Ca^{2+}pump sensitivity should be investigated over a similar age range.

The present work further indicates that the direct effect of caffeine in post mature muscle is not significantly affected by whole animal body mass. The give increase in whole animal body mass, largely attributed to greater adipose tissue, did not significantly affect the direct response of caffeine in 50-week

diaphragm muscle. This subsequently indicates that increased whole animal adipose tissue, and likely increase in muscle fat content, has little effect on skeletal muscle calcium handling properties. The effect of obesity on the direct muscle mechanical properties and the response to caffeine is an area that requires further investigation.

Conclusion

The present study is the first to assess the ergogenic properties of caffeine on isolated skeletal muscle performance from development to post maturity. The direct treatment of mouse muscle with, physiologically relevant, 70μM caffeine significantly potentiated the maximal power output of mouse EDL and diaphragm muscle independent of age. The ergogenic benefit of caffeine was significantly reduced in the older age groups but the effect may still be substantial to evoke significant enhancements in muscular performance. Caffeine also had a reduced effect on skeletal muscle in young mice, which brings into question the ergogenic benefit of caffeine in this population.

9. General Conclusion

Evidence presented in the present thesis provides an in-depth understanding of the ergogenic effects of physiologically relevant concentrations of caffeine when acting directly on skeletal muscle.

A summary of the unique and key findings of this thesis:

1. There was no dose dependent effect of caffeine on mouse soleus and EDL muscles at physiological levels; 50-70μM concentrations must be used to potentiate acute maximal force production. **(Chapter 4)**

2. 70μM caffeine induced a greater ergogenic effect in mouse muscle with a predominantly slower fiber type (i.e. in soleus compared with EDL). **(Chapter 4)**

3. In mouse EDL and soleus muscle, the caffeine induced force potentiation was not significantly affected by stimulation frequency. **(Chapter 4)**

4. At the skeletal muscle level there were inter-individual differences in response to caffeine with a division between responders and non-responders that cannot be related to caffeine habituation as these mice did not receive caffeine in their diet. **(Chapter 4)**

5. There was a fiber type specific effect of 70μM caffeine on mouse soleus skeletal muscle fatigue resistance. Maximally stimulated muscle fatigued faster with caffeine treatment in contrast to sub maximally stimulated muscle where endurance was improved by caffeine treatment. **(Chapter 5)**

6. There was no direct ergogenic effect of physiologically relevant concentrations of taurine on mouse soleus muscle performance, nor did taurine combined with 70µM caffeine potentiate the effect of 70µMcaffeine treatment alone. **(Chapter 6)**

7. The change in mouse skeletal muscle mechanical properties in development and ageing were muscle specific and are likely to depend on anatomical function and location and predominant fiber type. **(Chapter 7)**

8. The age related reduction in maximal muscle force exceeds the reduction in muscle power output when examined using isolated mouse EDL and diaphragm preparations. **(Chapter 7)**

9. The alterations in fatigue resistance of mouse skeletal muscle from development and with increasing age is likely to be more complex than first realized. Although fatigue resistance is muscle fiber type dependent, the present evidence identifies a four stage process whereby for both mouse EDL and diaphragm muscles: muscle in the development stage had the greatest fatigue resistance; fatigue resistance was reduced when the muscle reaches maturity; the muscle then became more fatigue resistant before a further secondary reduction in fatigue resistance within the oldest age group. **(Chapter 7)**

10. The ergogenic effect of 70µM caffeine was reduced in developing and with increasing age in mouse EDL and diaphragm muscles, which is likely a result of a deterioration in the performance of excitation contraction coupling. **(Chapter 8)**

The implications of the results of this thesis suggest that the consumption of caffeine in physiological doses can significantly enhance skeletal muscle performance. This coupled with the potential reported central nervous system and metabolic effects may result in large increases in sporting performance (Graham *et al.* 2001; Magkos & Kavouras, 2005; Davis & Green, 2009). As a result, the effects of caffeine abuse in a sporting context should be more closely considered with particular reference to the inclusion of caffeine on the World Anti-Doping Agencies' prohibited list. The present evidence suggesting an individualised ergogenic response to caffeine adding further weight to this debate. In essence caffeine use in sport may evoke a large increase in performance in some athletes' whilst in others the benefit is reduced or no effect is present. Subsequently, large responding performers will receive a legal performance boost that cannot be equally matched among all competitors. The remaining section of the discussion will focus on key aims of the thesis, which were to measure the direct effects of caffeine on skeletal muscle performance.

The present findings indicate that physiological doses of caffeine have a fiber type specific effect. Such findings have also been previously demonstrated using high concentrations of caffeine that would be toxic for human consumption (Fredholm *et al.* 1999).The present findings infer that the caffeine response is enhanced in muscles with a predominately slower phenotype. Subsequently this response may induce an amplified ergogenic effect of caffeine during prolonged submaximal activities that have a greater reliance on oxidative fibers. Interestingly the present results highlight that the magnitude of the caffeine effect is independent of muscle activation intensity. This therefore suggests that although the SR concentration of Ca^{2+} will be greater during submaximal intensity, the caffeine-induced release of Ca^{2+} is in some way limited. The mechanism limiting further calcium release needs to be elucidated to better understand the reasons underpinning the present study's findings.

The present findings also highlight the complex relationship between the ergogenic effect of caffeine and fatigue resistance. Findings provide further insight to the conclusions of James *et al.* (2005) suggesting that in muscle with a relatively slow fiber type composition, time to fatigue is reduced when the muscle is

stimulated maximally but prolonged when a submaximal intensity is used. This effect is attributed to the ability of the muscle to better handle increased intramuscular Ca^{2+} concentration at these lower stimulation intensities. I.e. when caffeine induces higher calcium release in maximally activated muscle the muscle is less able to handle the extra caffeine and fatigue resistance is compromised.

In conjunction with an increased performance of locomotory muscle, the present evidence demonstrates that 70μM caffeine might also be used to enhance acute power output of the diaphragm. Therefore, caffeine may play a role in increasing respiratory performance and subsequent interaction of the discussed effects may provide further explanation to the body of evidence that suggests a greater ergogenic effect of caffeine in prolonged endurance based actives (Graham *et al.* 2001; Davis & Green, 2009). In light of the performance enhancements demonstrated in the present studies, the use of caffeine as a training aid should be considered. Promoting work at a higher intensity and for a prolonged duration would allow an amplified training stimulus. In light of this it is important to consider personal regulation of caffeine consumption due to the potential desensitising to its effects and the health implications of long-term caffeine use. Bell and McClellan (2002) demonstrated that the ergogenic benefit of caffeine was greater in subjects that did not regularly consume caffeine, suggesting habituation to long term exposure. Furthermore habitual daily use of caffeine exceeding 500-600mg (4-7 cups of coffee) is believed to result in a significant health risk and sustained abuse may result in 'caffeinism' (Newrot *et al.* 2003). This is characterised by a spectrum of clinical symptoms ranging from anxiety and irritability, insomnia, muscle tremors, dieresis, arrhythmia, tachycardia, elevated respiration rate, and gastrointestinal disturbances (Newrot *et al.* 2003). Contrary to common belief recent articles suggest that caffeine does not promote ventricular arrhythmia and myocardial infraction, suggesting the evidence examining the effects of caffeine on the cardiovascular system is inconclusive (Peters *et al.* 2001; Katan & Schouten, 2005). A review by Newrot *et al.* (2003) concluded that for a healthy adult population, moderate daily caffeine intake at doses up to 400mg per day (equivalent to 6 $mg.kg^{-1}$ per body weight in 65 kg person) is not associated with such adverse effects (Newrot *et al.* 2003). Interestingly this is within the range of

caffeine doses employed within the majority of whole body human research examining the ergogenic effects of caffeine a sample of which is outlined in Tables 1.4.1 and 1.4.1.

Many energy drinks contain a range of compounds that their manufacturers suggest will actively improve human exercise performance. We have demonstrated that physiological concentrations of caffeine can directly enhance skeletal muscle performance. However, many energy drinks also contain high concentrations of taurine. Despite evidence suggesting enhanced skeletal muscle function following chronic supplementation, the acute effect of taurine, such as that delivered by energy drinks, has received very little attention. Evidence from the present work demonstrates that taurine, at a physiological concentration, fails to elicit a direct effect on skeletal muscle function (Chapter 6). Furthermore, in contrast to suggestions from past literature (Geib *et al.* 1994; Steele *et al.* 1990), using non-physiologically relevant concentrations of taurine and caffeine, an interaction of these ingredients does not occur and taurine fails to potentiate the effect of caffeine alone.

The body of evidence that examines the effect of caffeine on performance (both *in vivo* and *in vitro*) is almost entirely based on subjects (both human and other mammals) that are around their age related physiological peak. As well as being the first to provide an in-depth examination of the mechanical properties of skeletal muscle from development and with increasing age, the present evidence also investigates the potential age related response to the ergogenic effect of caffeine. Initially the findings from this thesis suggest that muscle development is likely to be related to predominant phenotype and physiological function, as diaphragm muscle matured faster than locomotory EDL muscle. The present evidence also highlights the complexity of the ageing process as mechanical properties of skeletal muscle deteriorate at different rates and this is also muscle dependent. The question as to which muscle ages faster is fundamentally unanswerable as the present evidence indicates a variation between the mechanical properties measured. Interestingly, diaphragm muscle shows a greater loss of maximal force and power compared to EDL. This is of significance as previous evidence suggests that muscles with a larger proportion of fast fiber types (such as EDL), would have a greater age related decrement in function

due to such fibers experiencing atrophy to a larger degree. Conversely, EDL muscle demonstrated a much greater deterioration in fatigue resistance compared to diaphragm muscle.

Additionally, the present evidence is the first to shed light on the equivocal findings on the effect of ageing on fatigue. The magnitude in this alteration varies, however a clear 4-stage pattern has been discovered. During the developmental stage muscle was at its most fatigue resistant, but fatigue resistance had decreased when maturity was reached. Following this, as the animal begins to age further, fatigue resistance increased before decreasing again. In the case of EDL this was significantly reduced in comparison to mature values but middle aged and mature fatigue resistance were similar in the diaphragm muscle. In contrast to previous evidence the present findings also indicate that the reduction in maximal muscle force exceeds the reduction in maximal muscle power output. As the ageing response was correlated to a decrease in EDL muscle mass, it is suggested that the enhanced reduction in power in human research is a consequence of the further interaction of atrophy and deterioration in neuromuscular innervation.

Having a greater understanding of the ageing response is important in the potential development of innovations to improve human health and quality of life. Ageing diaphragm muscle is likely to significantly affect cardiorespiratory function and may contribute to COPD or related symptoms in the elderly. Furthermore with falls relating to greater rates of morbidity and mortality in elderly citizens, the importance of understanding ageing effects on peripheral muscle performance is of equal importance (Aschkenasy & Rothenhaus, 2006).

The result of this age and muscle dependent alteration in mechanical properties greatly affects the direct force potentiating properties of 70µM caffeine. Caffeine evoked the greatest potentiation of maximal force when muscle was in the range of peak maturity. During stages of development and later stages of ageing, in the context of the present study, the ergogenic effect of caffeine was greatly reduced. Alterations in the magnitude of the effect of caffeine infer age related changes in excitation contraction coupling. Although the effect of caffeine was significantly reduced in older muscle, the potential

ergogenic effect may still provide a significant enhancement in the physical performance of elderly citizens and subsequently may have a role to play in increasing quality of life and rehabilitation program performance. A more senior population may be long term habitual caffeine users and as such the potential reduction in caffeine sensitivity should be considered here. Furthermore, as with all individuals that use caffeine as a means to improve physical performance a degree of self-regulation is essential.

10. Future Work

The group of studies in the present thesis assess a number of questions regarding the effects of physiological concentrations of caffeine on isolated mouse skeletal muscle performance and the changes in mechanical properties that occur with age. In light of these findings a number of further questions have been raised which could structure the future direction of caffeine and ageing research.

- Is the ageing effect and subsequent change in the ergogenic effects of caffeine different in ageing male mice? The speed of ageing in male mice is likely to be different to that in female mice due to differences in gender related hormone secretion. Subsequently it would be interesting to repeat the studies outlined in chapters 4 and 5 in male mice to make a direct comparison to the results in female mice.

- How much can increased physical activity offset the ageing response and potentially improve the ergogenic effect of caffeine? Training 'middle aged' mice and comparing the mechanical properties of their skeletal muscle with an untrained group would enable us to assess if the direct effect of training on skeletal muscle performance can counteract the ageing response. Maintaining muscle properties might also affect the direct ergogenic effect of caffeine in aged muscle, therefore examining the caffeine response in trained post mature muscle would allow us to quantify if the ergogenic effect is trainable and as such is it possible to enhance the individual caffeine benefit.

- How much is the effect of ageing on skeletal muscle affected by apparent differences in the quantity of body fat? A further area of interest would be to examine how much of the age related changes in muscle mechanical properties are a true effect of ageing and what extent obesity plays

in the loss of muscle function. Analysis of the results from the present work suggest that middle aged mice with a lower body mass-produced significantly higher diaphragm PO per g body mass compared to those with a higher body mass (Fig 10.1; TTest $p<0.001$).There was no significant difference in mean diaphragm work loop PO per kg muscle mass between mice with a high body mass compared to those with a lower body mass (Fig 10.1; TTest $p=0.17$).However the trend for decreased muscle PO is evident and is an area that needs further analysis. Increased body weight could affect the amount of exercise the mouse performs in each cage further increasing the age related deterioration in muscle performance. Changes in daily activity could be monitored in each mouse cage during ageing.

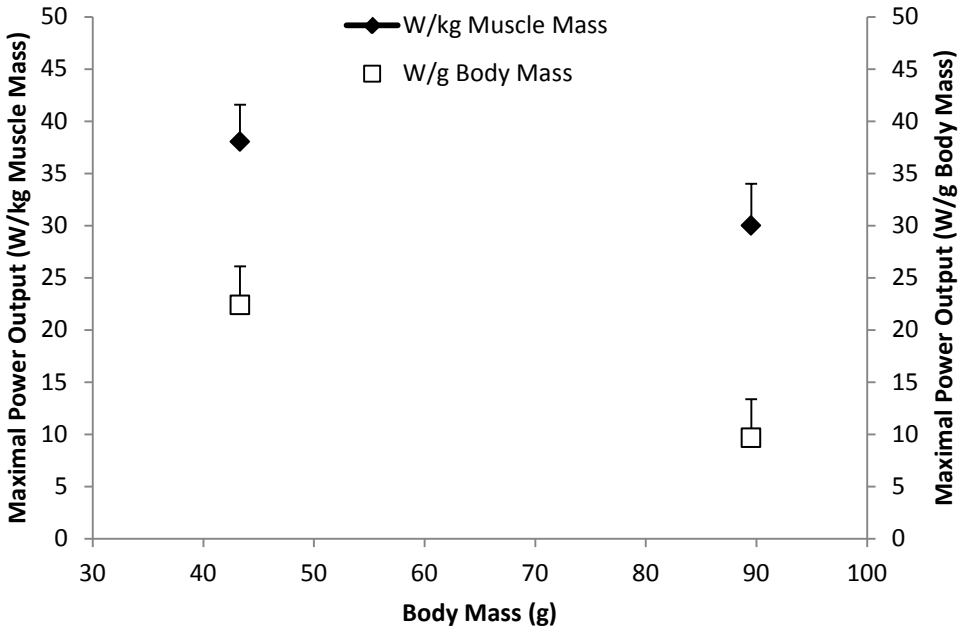


Figure 10.1 – A comparison of mean maximal work loop power output of diaphragm muscle of middle aged mice between those with a high body mass and those with a low body mass. [Data represented as mean ± SE: n=5 in each case).

- The effect of caffeine on exercise performance in elderly humans. Presently there are a limited number human studies that assess the ergogenic effect of caffeine in elderly whole body performance. This would be an interesting area of research as potentially the ergogenic benefit is

likely to be greater if the direct skeletal muscle effects are combined with the potential CNS stimulant effects.

11. References

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